THE TOHOKU JOURNAL OF EXPERIMENTAL MEDICINE

Vol. 73. No. 2, January 25, 1961

OHIO STATE UNIVERSITY
JAN 9 1962
LIBRARY

EDITORIAL BOARD
T. KATO K. MOTOKAWA M. WADA

WITH COLLABORATION OF

K. AKAZAKI, Ts. ARAKAWA, T. EBINA, T. FUJITA, Y. HAYASHI, S. IINO, T. ISHIBASHI, N. ISHIDA, T. ISHIKAWA, M. ITO, K. IWATSUKI, S.-T. KATSURA, G. KIKUCHI, N. KIRISAWA, Y. KOGA, S. KONDO, T. KUMAGAI, T. KUROKAWA, M. KUROYA, K. KUSHIMA, T. MURAKAMI, M. MUTO, T. NAKAMURA, F. NAKAZAWA, S. NASU, S. OSATO, T. SANO, A. SATO, S. SATO, M. SEGI, T. SHIMAUCHI, H. SETO, T. SHINODA, T. SUGIYAMA, N. SUWA, T. SUZUKI, E. TAKAHASHI, Y. TAKAHASHI, M. TERASAKA, T. TORIKAI, Y. TUIKI, R. URA, S. YAMAGATA

PUBLISHED BY
THE TOHOKU UNIVERSITY
SENDAI, JAPAN

The Tohoku Journal of Experimental Medicine is an official organ of the Tohoku University for the publication of records of original research in all branches of experimental medicine, but accepts also papers from sources other than the University, domestic as well as overseas, under certain limitations, given below.

Two to three volumes, each containing four numbers, are issued a year. Each volume will consist of approximately 400 pages.

The articles are written in English, French or German.

The subscription price is $5.00, foreign $5.50, per volume, postpaid, and should be sent to the MARUZEN Co., LTD., Tokyo or CHARLES E. TUTTLE Co., RUTLAND, VERMONT, U.S.A.

Papers and correspondence regarding exchange of publications, etc. should be addressed to the Medical Library, Tohoku University, Kita-yoban-tyo, Sendai.

Notice to Contributors

Papers will not be accepted for simultaneous publication or those which have been previously published in the periodicals written in English, French, German or Italian. All articles from Laboratories must be approved by the director of the originating laboratory. Contributions should be ordinarily limited in length to 10 printed pages. Every paper must be accompanied by a brief summary.

The manuscripts should be typewritten in double spacing, and carefully revised before submission to the Editor. The original, not carbon, copy should be sent. Bibliographies should give the name of the author, the name of journal or book (with edition & place of publishing), the year, the volume and the page.

Figures should be ready for photographic reproduction, diagrams should be drawn in Indian ink and plain white or fine blue-lined paper only should be employed; letters, numbers etc. should be written in pencil. The reduction desired should be clearly indicated on the margin of the drawing. The Journal cannot accept illustrations which when reproduced for printing exceed the size of a Journal page. Authors should indicate on the manuscript the approximate position of the text figures and tables.

All the drawings and tables should be marked with the author's name. They should be packed plate, carefully protected from injury, and insured.

Proofs will not be submitted to authors overseas unless they express a desire to see them, or unless the editors think it necessary. Overseas authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that errors need not arise.

Authors receive 100 reprints of articles at cost.

Tohoku J. Exper. Med., 1961, 73, 103–117

Electromyographic Studies on Urinary Tract

II. Electromyographic Study on the Genesis of Peristaltic Movement of the Dog's Ureter

By

Tsuneo Shiratori and **Hideo Kinoshita**

From Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, August 27, 1960)

INTRODUCTION

Since the physiological studies on the ureteral peristalsis first undertaken systematically by Engelmann, there have been many researches over a wide scope in the field to date, and yet there are many a point left in the dark concerning the mechanism of appearance of peristaltic movement in the ureter.

As works on the genesis of peristaltic movement in the ureter we have the works of Engelmann[1], Protopopow[2], Alksne[3] and Penfield[4] on vital ureters, and of Sokoloff *et al.*[5], Satani[6], Boulet & Gley[7], Hryntschak[8], Gruber[9], Wu[10], Nobuta[11] and Takagi[12] on excised ureters, but all these have relied on ocular or kymographic observations, only Sleator & Butcher[13] having availed of electromyography in their study. In our previous report[14], the normal peristaltic movement of the ureter between the pyeloureteral junction and the ureterovesical junction was observed by means of EMG. In this report, we will report on the results of EMG study of the ureteral peristalsis following intravenous injection (i.v.) of xylocaine and novocaine and periureteral injection of xylocaine and in the next report, on those following pinching and cutting of the ureter.

METHODS OF OBSERVATION AND EXPERIMENT

In a laboratory kept at 15–25°C, 38 adult dogs were fixed in dorsal position, and laparotomized under anesthesia with 10 mg./kg. of thiopental sodium, and their entire ureters between the pyeloureteral and ureterovesical junctions or a part of them required for the experiment were laid bare.

Electrodes of silver needles were inserted into the ureteral wall and fixed at the distance of 2–4 cm., so as to be movable with the peristalsis. One, two or three pairs of such electrodes were used per ureter, to make bipolar leading.

白鳥常男，木下秀雄

A Poly-Viso, Sanborn Co. was used to recording action potentials. The measuring methods of wave interval, propagation velocity, amplitude, duration and wave pattern were the same as those described in our previous report.[14]

EXPERIMENTAL RESULTS

I. EMG of ureter between the pyeloureteral and the ureterovesical junctions in normal six dogs

Under normalcy, the discharges due to the ureteral peristaltic movement, as recorded by bipolar leading, always begin at the pyeloureteral junction and reached down to the ureterovesical junction (Fig. 1), and we found none of the discharges stopping midways, arising midways or running antiperistaltically.

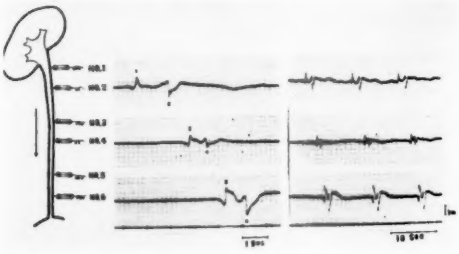


Fig. 1. Ureteral EMG led off by 3 pairs of bipolar leading.

TABLE I. Propagation Velocity of Peristaltic Movement at Each Part of Ureter

No.	Upper 1/3	Middle 1/3	Lower 1/3
1	13.3~16.6 mm/sec	27.0~31.2 mm/sec	21.4~24.2 mm/sec
2	27.0~29.3	29.3~30.7	25.6~27.7
3	26.3~29.4	29.4~31.2	25.0~26.3
4	18.9~20.4	32.6~34.1	27.4~29.7
5	26.2~26.8	29.5~31.4	25.0~27.0
6	13.3~30.8	27.0~34.1	23.5~29.7

These discharges showed the frequency of 4–15 w/min. and their velocities, as shown in Table I, were faster in the mid-part than in the upper and the lower parts of the ureter.

Graves[15] had reported that the propagation velocity of peristaltic discharges in the ureter of rabbit was the fastest in its upper part and the slowest in the lower part and Sleator & Butcher[13] found the velocity faster in the lower than in the middle part in some of his cases.

The amplitude, the duration and the wave pattern are apt to change by the electrical contact between the electrodes and the ureter, so that a comparison of these data obtained from three parts of the ureter was impossible, but our observations resulted in amplitudes of 0.4–2.1 mV., 0.4–1.6 mV. and 0.4–1.8 mV., in durations of 0.3–0.75 sec., 0.3–0.7 sec. and 0.3–0.7 sec., and in wave patterns of I or II, I or II and I or II as classified in our previous report[14], in the upper, the middle and the lower part of the ureter, respectively.

II. EMG following i.v. injection of xylocaine and novocaine

1) Following i.v. injection of 0.4 mg/kg of 2% xylocaine solution in 5 cases, the frequency increased markedly, as shown in Fig. 2, and a tendency to group formation of the discharges was observed in the earliest stage of the increase in frequency, and the shortest and most regular intervals were found at the peak of the increase.

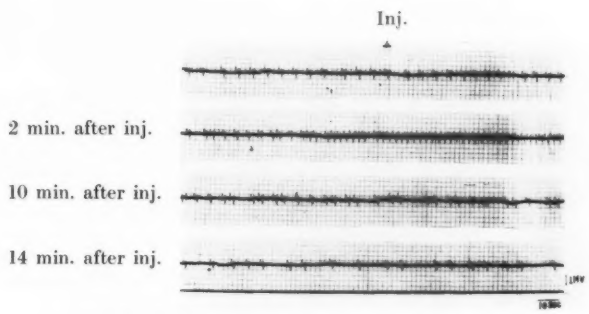


Fig. 2. Ureteral EMG following i.v. administration of 2% xylocaine, 4 mg./kg.

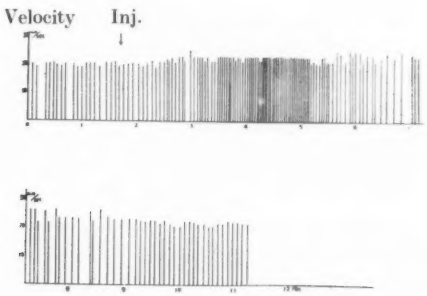


Fig. 3. Propagation velocity of peristaltic discharges by i.v. injection of 2% xylocaine, 4 mg./kg.

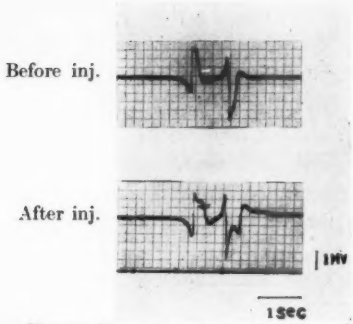


Fig. 4. Change in wave pattern by i.v. administration of 2% xylocaine, 4 mg./kg.

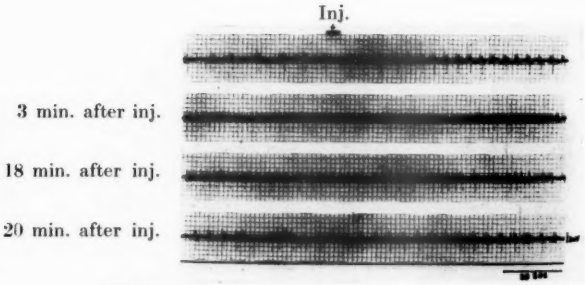


Fig. 5. Ureteral EMG by i.v. administration of 2% xylocaine, 10 mg./kg.

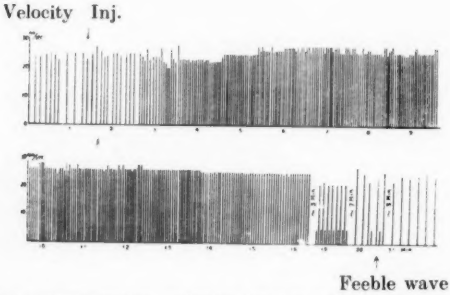


Fig. 6. Propagation velocity by i.v. administration of 2% xylocaine, 10 mg./kg.

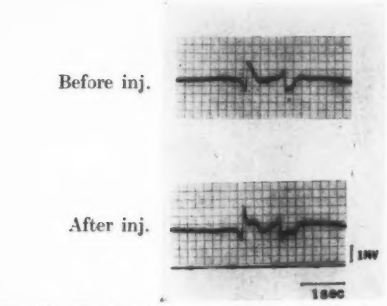


Fig. 7. Change in wave pattern in the above cases.

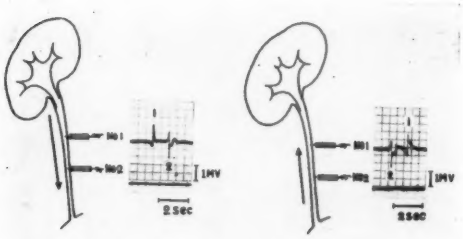


Fig. 8. Normo- and anti-peristaltic discharges.

Another grouping of the discharges appears in the stage when the frequency falls off gradually, and about 10 min. after the injection, the frequency comes down to the original level.

The velocity of the discharges also rose after the injection, as shown in Fig. 3, where the time elapsed is plotted in min. on the abscissa and the velocity in mm/sec. on the ordinate, the length of an inscribed vertical bar represents the velocity and the intervals between the bars the discharge intervals.

As shown in Fig. 4, the wave pattern sometimes passed over from the original Type II into Type I and in some cases of original Type I (cf. our previous report[14]) minor negative deflection became more apparent, after the injection.

The duration of a discharge lengthened slightly by the injection from 0.2–0.32 sec. to 0.3–0.44 sec. but the amplitude showed no change by the injection.

Following i.v. injection of 10 mg./kg. of 0.2% xylocaine in 12 dogs, as shown in Fig. 5, the frequency of discharges increased markedly in all the experiments to 20–34 w/min. in 2–10 min., but later on dropped to the normal level.

No grouping of the discharges was observed in the initial stage of rising frequency and the peak of the frequency was attained rather very soon; the discharge intervals were uniform at this peak and in the stage before normalization

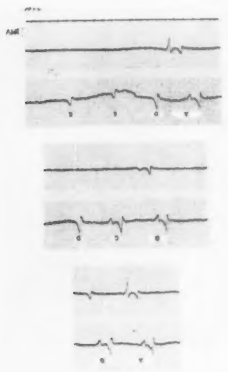


Fig. 9. Feeble waves occurred by i.v. inj. of 2% xylocaine, 10 mg./kg. (led off with 4 electrodes).

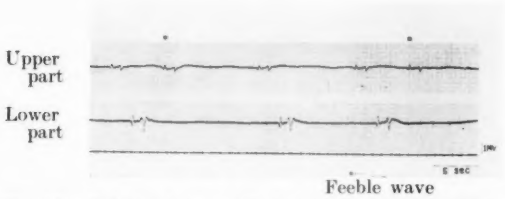


Fig. 10. Relation between discharge intervals and occurrence of feeble wave (led off with two pairs of bipolar leading).

Fig. 11. Ureteral EMG by i.v. administration of 2% novocaine, 4 mg./kg.

a tendency to grouping discharge formation became apparent.

The propagation velocity rose after the injection, as shown in Fig. 6.

The change of wave pattern (Fig. 7) was similar to that observed in the proceeding experiment, Type I and negative minor deflections becoming more frequent.

The duration lengthened from 0.25–0.4 sec. before the injection to 0.35–0.5 sec., but the amplitude remained unchanged around 0.5–2.0 mV.

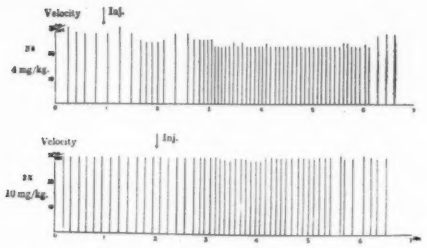


Fig. 12. Change of propagation velocity by i.v. administration of 2% novocaine, 4 or 10 mg./kg.

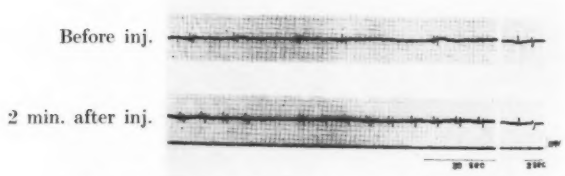


Fig. 13. Ureteral EMG by i.v. administration of 2% novocaine, 10 mg./kg.

In two of these cases,ten antiperistaltic discharges were found occurring in a series transiently in the first stage of increasing frequency. These discharges, as shown in Fig. 8, showed no difference from the normoperistaltic discharges in velocity, amplitude, duration and wave pattern, though reverse in their phases.

In one of the 5 cases injected with 4 mg./kg. and 5 of 12 cases injected with 10 mg./kg. of xylocaine, as shown in Fig. 9, discharges fading out midways before reaching the lower electrode were observed. We wish to call them "feeble waves", and it was notable of these feeble waves that they occurred at short intervals before or after normoperistaltic waves, as shown in Fig. 10.

2) Following injection of 2% novocaine solution in the dose of 4 mg./kg. in 5 cases, the frequency of the discharges rose to about twice in 2–10 min. and then fell off gradually to the preinjection value and the other characteristics of the discharges were little affected by the injection (Figs. 11, 12 and 13).

From the above findings, it was concluded that i.v. injection of the sensory depressants, xylocaine and novocaine caused acceleration of the peristaltic movement of the ureter.

III. EMG following periureteral injection of xylocaine

In the next place, we made comparative examinations of the ureteral EMG following injection of 40 mg. each of xylocaine into the tissues around the

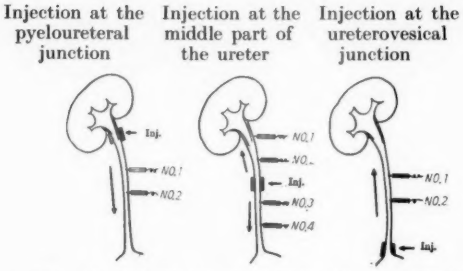


Fig. 14. 2% xylocaine solution 40 mg. was injected periureterally at each part of the ureter.

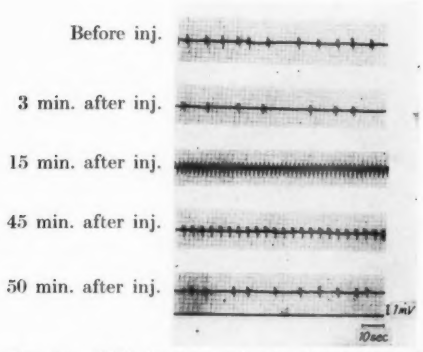


Fig. 15. EMG by the periureteral injection of 2% xylocaine 40 mg. at the pyeloureteral junction.

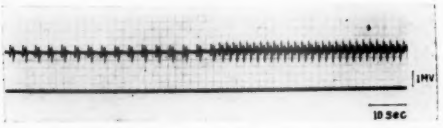


Fig. 16. Steplike change of discharge intervals in the cases where 2% xylocaine 40 mg. was injected periureterally at pyeloureteral junction.

pyeloureteral junction, the middle part of the ureter and the ureterovesical junction (Fig. 14).

1) Following injection around the pyeloureteral junction

In 5 cases, normoperistaltic discharges continued appearing after the injection (Fig. 15, left side), but their frequency, as shown in Fig. 15, dropped for a while, but then rose gradually to the maximum in 20–35 min., then fell off to the original

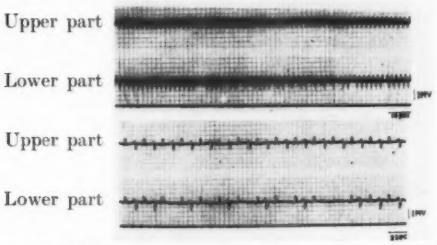


Fig. 17. Feeble waves occurring after periureteral injection of 2% xylocaine at pyeloureteral junction.

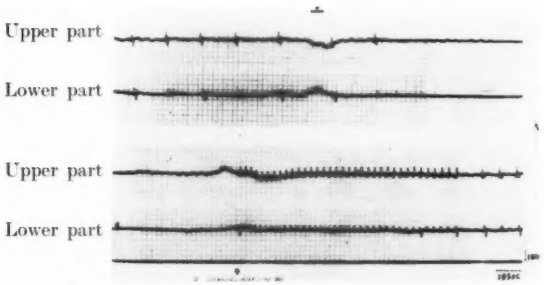


Fig. 18. Occurrence of normo- and anti-peristaltic discharges in the cases, 2% xylocaine 40 mg. was injected periureterally at the middle part of the ureter.

level in 70–75 min. The intervals between discharges were found to shorten abruptly with this increase in frequency, as shown in Fig. 16. The distal electrode was inserted near the ureterovesical junction, for assuring whether the discharges reached thus far; in 3 of the 5 cases, it was found, as shown in Fig. 17, that feeble waves arising in the upper part but not reaching the lower end of the ureter occurred in the stage of recovery after the stage of notably increasing frequency.

The velocity of the discharges was found to rise in the stage of transient decrease of frequency, to slacken at the stage of increasing frequency and to normalize as the frequency returned to the original level. The duration and the amplitude of discharges rose slightly at the stage of increasing frequency, while the wave pattern Type I before the injection was accompanied by occurrence of noticeable minor negative deflections at the stage of transiently reduced frequency, turned into Type II after the continued rise of the frequency and returned to Type I at the stage of normalized frequency.

2) Following injection around the middle part of the ureter

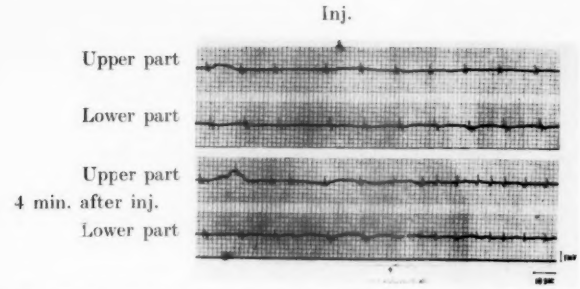


Fig. 19. The same phenomenon as in Fig. 18. was found in the other case.

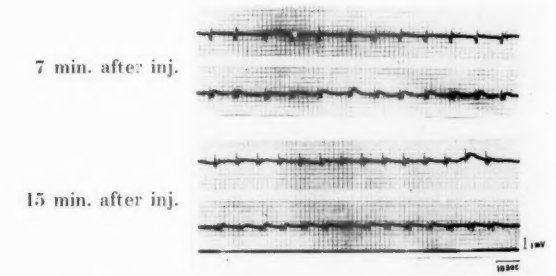


Fig. 20. EMG by the periureteral injection of 2% xylocaine 40 mg. at the middle part of th ureter.

In 4 of 7 cases, as shown in Fig. 18, we saw the discharges entirely suppressed for 20–100 sec., 20–38 sec. after the injection: thereafter simultaneous antiperistaltic and normoperistaltic discharges set off upwards and downwards from the injected part in the ureter, respectively. These discharges had the frequency of 2–24 w/min., appeared at the regular intervals of 2.4–2.8 sec. and continued for 62–150 sec., thereafter returned to the spontaneous normoperistaltic discharges before the injection.

In the other 3 cases, as shown in Figs. 19, 20, without the stage of suppressed discharges, the simultaneous anti- and normo-peristaltic discharges began to occur in 107–223 sec. after the injection, at the frequency of 5–10 w/min. and continued for 9–14 min.

In all the above cases, feeble waves were found mingled with the normoperistaltic discharges running downwards from the injected part. The particulars about the spontaneous preinjectional normoperistaltic discharges and the anti- and normo-peristaltic discharges appearing after the injection were as summarized in the following table.

Table II. Local Differences

	Propagation velocity	Amplitude	Duration	Wave pattern
Before injection				
	mm/sec.	mV.	sec.	Type
In upper ureter	22.2–28.5	0.7–1.4	0.3–0.5	I – II
In lower ureter	20.6–23.0	0.8–1.1	0.4–0.5	I – II
After injection				
In upper ureter (antiperist.)	25.0–28.6	0.5–1.4	0.35–0.4	I – II
In lower ureter (normoperist.)	20.0–22.2	0.7–1.1	0.45–0.6	I – II

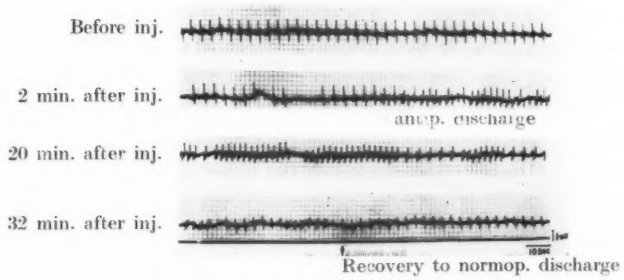


Fig. 21. EMG by periureteral injection of 2% xylocaine 40 mg. at ureterovesical junction.

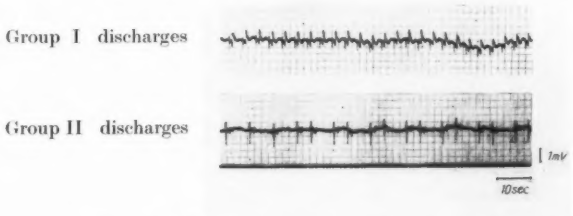


Fig. 22. Intervals of the antiperistaltic discharges by periureteral injection of 2% xylocaine 40 mg. at ureterovesical junction.

3) Following injection in the ureterovesical junction

The urinary bladder of 5 dogs were cut open, the vesical orifice of the ureter was exposed and the injection was made in the surrounding connective tissue.

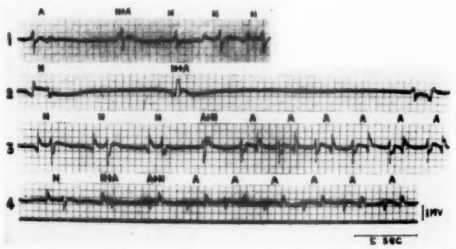
Before the injection, spontaneous normoperistaltic discharges of 3–11 w/min.

were observed, and after the injection, in 1–5 min. antiperistaltic discharges of 10–28 w/min. began to appear and continued for 2–32 min. before returning to the spontaneous normoperistaltic discharges, as shown on the right of Fig. 14, and Fig. 21. These antiperistaltic discharges, as shown in Fig. 22, were of either the uniform interval type (the Group I according to Shiratori *et al.*) or grouped type (ditto, the Group II).

At the stages of transition of normoperistaltic into antiperistaltic discharges and of anti- into normo-peristaltic discharges, we observed mixed occurrence of discharges in both ways in some cases, but in the other, no such coexistence of reverse discharges was seen.

These postinjectional antiperistaltic discharges differed little from the preinjectional normoperistaltic discharges in propagation velocity, amplitude, duration and wave pattern.

4) As control tests, 2 cc of physiological saline was injected into the surrounding tissues of the pyeloureteral junction, the middle part of the ureter and the ureterovesical junction in 5 cases each. The behavior of occurrence, the propagation velocity, the duration, the amplitude and the wave pattern of the peristaltic discharges were not all affected by the injection.



N : Normoperistaltic D. A : Antiperistaltic D.

Fig. 23. Normo- and anti-peristaltic discharges were found simultaneously.

In the series of experiments above, when normo- and anti-peristaltic discharges appear simultaneously, we may find whether a positive (normo. plus anti.) or a negative (anti. plus normo.) discharge precedes by placing two electrodes at this site, as shown in Fig. 23, according to whether the normo- or antiperistaltic discharges arrive first; in such cases, we found that when the discharges taken up by the two electrodes have extremely shortened intervals, interference waves might be observed.

After the appearance of such N. plus A. and A. plus N. waves, the normo- and the anti-peristaltic discharges seemed to have come into collision between the two electrodes, and the discharges to have neutralized after the collision and

TABLE III. Direction of Discharge, Frequency and its Continuing Time by the Periureteral Injection of 2% Xylocaine 40 mg.

Localization of the inj.	Direction	Frequency in w/min.	Direction	Frequency in w/min.	Time of continuing
Pyeloureteral junction	Normo.	2–15	Normo.	20–37	18–75′
Middle part of the ureter	Normo.	3–15	Normo. & Anti.	5–24	1′2″–14′30″
Ureterovesical junction	Normo.	3–11	Anti.	10–28	2–32′

refused leading.

In short, we have observed that series of discharges, either normo- or anti-peristaltic, emanated from the site of xylocaine injection around any part of the ureter. Besides, it was found that when normo- and/or anti-peristaltic discharages occurred following injection into the tissues around the middle part of the ureter and the ureterovesical junction, the spontaneous normoperistaltic discharges were suppressed.

The frequency and the continual time of the discharges, as shown in Table III were largest in those arising in the pyeloureteral junction, followed by the values of those arising in the ureterovesical junction and in the middle part of the ureter, suggesting that any part of the ureter may be thrown into an excited state by periureteral injection, leading to peristaltic movement. The excitation following injection of the same dose of xylocaine was the most intense at the pyeloureteral junction, next in the ureterovesical junction and the smallest in the middle part of the ureter.

Sleater & Butcher[13] injected 2% xylocaine and 2% novocaine solutions around the dog's ureters and electromyographically observed that unilateral or bilateral peristaltic discharges of 15–30 per min. in frequency occurred and continued for several minutes, while Penfield[4] macroscopically observed peristaltic movements upwards and downwards from the site of rabbit's ureter on which he dripped barium chloride, but these results were obtained by tampering with the middle part of the ureter alone.

From our observation on the occurrence of anti- and normo-peristaltic discharges from the middle part and anti-peristaltic discharges from the ureterovesical junction, following injection of xylocaine and the disappearance of the normo-peristaltic discharges from the pyeloureteral junction while the above non-normal discharges were being emitted, we can deduce that the excitation at the site surpasses the excitation in the pyeloureteral junction in intensity, the part of highest excitation determines the direction of peristalsis and the normal function of the pyeloureteral junction in causing peristaltic movement is inhibited in the

interim.

Gruber[9] reported that an administration of epinephrine or urea at one end of excised ureters caused predominance of the administered site and a temporary reversion of the direction of peristalsis and remarked that nicotine and acetylcholine also could cause such a reversion; the reversion of peristaltic movement following our administration of xylocaine also seemed to indicate a similar effect of xylocaine to the drugs he used.

SUMMARY

EMG of the ureter between the pyeloureteral and the ureterovesical junctions of 38 adult dogs was observed under normalcy, and following i.v. injection of 2% xylocaine and 2% novocaine solutions and periureteral injection of 2% xylocaine solution.

The results obtained were as follows:

1. The normal ureteral peristaltic discharges always arise at the pyeloureteral junction and reach the ureterovesical junction, and the propagation velocity of these discharges was higher in the middle part of the ureter than in its upper and lower parts.

2. Following i.v. injection of 4 mg./kg. and 10 mg./kg. of 2% xylocaine solution, 1) the discharge frequency was definitely increased, 2) the velocity of propagation was accelerated, 3) the intervals between discharges were markedly shortened, groups of discharges of Shiratori *et al's*[14] Group I or II being formed, 4) Type II wave pattern changed into Type I and Type I waves were accompanied by perceptible minor negative deflections and 5) in some cases, feeble waves fading out midways were found emerging in the stage when the frequency after reaching the maximum tended to come down to normal level.

3. Following i.v. injection of 4 mg./kg. of 2% novocaine, the frequency increased and the velocity of propagation showed down, but after injection of 10 mg./kg. of the same solution, only the frequency rose a little, the propagation velocity, the amplitude, the duration and the wave pattern remaining almost unaffected.

4. The interesting finding that the sensory depressans, xylocaine and novocaine stimulate the ureteral peristaltic movement was obtained.

5. Following injection of xylocaine around the pyeloureteral junction, a series of normoperistaltic discharges was emitted when the site of injection was around the middle part of the ureter, simultaneous antiperistaltic discharges upwards and the normoperistaltic discharges downwards from the site occured serially, and when the injection was administered in the surrounding tissue of the ureterovesical junction, a series of upward antiperistaltic discharges was emitted.

The frequency and the duration time of these serial discharges were the largest in the pyeloureteral junction, next in the ureterovesical junction and the lowest in the middle part of the ureter.

6. It was concluded that the peristaltic movement of the ureter was determined in its direction by the site, normoperistaltic movement alone arising at the pyeloureteral junction, antiperistaltic movement alone at the ureterovesical junction and normo- as well as anti-peristaltic movements at any other middle part of the ureter.

References

1) Engelmann, T.W., Arch. f. d. ges. Physiol., 1869, **2**, 243.
2) Protopopow, S.A., *ibid.*, 1897, **66**, 1.
3) Alksne, J., Folia. Urolog., 1907, **1**, 338.
4) Penfield, W.G., Amer. J. Med. Sc., 1920, **160**, 36.
5) Sokoloff *et al.*, Arch. f. d. ges. Physiol., 1881, **26**, 464.
6) Satani, Y., Amer. J. Physiol., 1919, **49**, 474.
7) Boulet et Gley., C.R. Soc. Biol., Paris, T. LXXIV, p. 1171, 1913. Cited by Takagi, M. (1933).
8) Hryntshak, T., Arch. f. d. ges. Physiol., 1925, **209**, 542.
9) Gruber, C.M., J. Urol., 1928, **20**, 27.
10) Wu, P.P.T., *ibid.*, 1933, **66**, 307.
11) Nobuta, S., Jap. J. Urol., 1926, **14**, 119. (Jap.)
12) Takagi, M., Acta Med., 1933, **7**, 831. (Jap.)
13) Sleater, W., Jr. & Butcher, H.R., Jr., Amer. J. Physiol., 1955, **180**, 261.
14) Shiratori, T. *et al.*, Tohoku J. Exp. Med., 1957, **55**, 882.
15) Graves, R. C. & Davidoff, L.M., J. Urol., 1923, **10**, 185.

Tohoku J. Exper. Med., 1961, 73, 118–126

The Origin of the Ponceau-fuchsinophilic Cells of the Human Adrenal Cortex and Their Progressive and Regressive Changes in the Fetal and Postnatal Periods

By

Nobuaki Sasano

Department of Pathology of Prof. N. Suwa, Tohoku University School of Medicine, Sendai, Japan

(Received for publication, August 29, 1960)

There has been a remarkable advance in recent studies of histological and functional zonation of the adrenal cortex. The structures and histological changes of the organ in reference to its functional siginificance have likewise been elucidated gradually.[10),11),14),16)] Much is thus known about the morphology of the fetal cortex of the adrenal and its possible physiological functions, of which androgen secretion is recognized for a long time.[1),2),3),12)] The first remark in this field was made by Vines[18)] (1933), who studied the correlation between fuchsinophil materials of the adrenal and androgen secretion. Later investigations of Carnes,[7)] Gersh and Grollman[8)] could not confirm androgen in the extracts of fetal adrenal. However, Zinsser and Zinsser[20)] considered the material, which was positive for ponceau-fuchsin staining, as ketosteroid-protein complex. In any way, it must be acknowledged that the fetal cortex secretes steroid in intra-uterine period and the ponceau-fuchsin granule is one of the indications of its functional activities. Though the granule is specific for the fetal cortex, little is known about the course of its development. According to Botella-Llusiá,[4)] Jones,[11)] Neumann,[13)] Rotter[15)] and other investigators, the fetal cortex is maintained by chorionic gonadotropin. But the process is not yet correlated to the fluctuation of the specific granules. The purpose of the present investigation is, therefore, to try to throw light on the problem by means of ponceau-fuchsin staining on a number of the adrenals obtained from human embryos and infants.

MATERIALS AND METHODS

The adrenals were obtained from 404 autopsy cases from 2 months old embryos to 6 months old infants as soon as possible after death. The organs were fixed in formalin solution, embedded in paraffin and sectioned to 5 μ in thickness. The sections were stained according to the original method des-

笹 野 伸 昭

cribed by Vines. The materials were strictly selected for examination, so that only those materials were evaluated, which were well stained by hematoxylin-eosin and had preferably little postmortem change. For embryological investigations embryos from 4 mm to 24 mm in length were available. The embryos were obtained by curettage in their perfect form, immediately fixed in formalin and embedded in paraffin. Ten μ thick horizontal serial sections were prepared and the sections, which included the adrenal, were stained after the above-mentioned method.

The results were evaluated as positive, when bright red staining granules or diffusely distributed substance discribed by Vines, Verzár and others were demonstrated. To ascertain that the material was sufficiently well preserved for staining, the result of Heidenhain's iron hematoxylin staining and Altmann-Kiyono's staining on the same material were taken into consideration. For a control examination, the adrenals of 27 autopsy cases ranging from the 7th month to the 64th year of age, were treated and stained by the same procedure.

RESULTS

Out of 404 examined cases, older than 2 fetal months, distinctive ponceau-fuchsin granules were demonstrated in 132 cases. In other 141 cases the results were negative, and the remaining cases gave uncertain or ambiguous reactions (Table I). The granules had their highest incidence in the third fetal month, being positive in 24 cases out of 28 (86%) and, after some fluctuations in the following months, disappeared with the fifth postnatal month. The rate of incidence

Table I. Data According to the Monthly Distribution of Results by Ponceau-Fuchsin Stain

Age		Case	+	+?	±	−	?
Fetal Month	2	4	3	0	0	0	1
	3	28	24	0	2	1	1
	4	20	11	0	3	6	0
	5	27	16	0	6	4	1
	6	53	13	3	13	21	3
	7	48	11	1	16	20	0
	8	44	12	1	12	19	0
	9	30	7	0	10	7	6
	10	57	17	3	15	19	
Postnatal Month	1	46	10	0	9	26	1
	2	16	4	0	4	8	0
	3	12	3	0	4	5	0
	4	7	1	0	5	1	0
	5	6	0	0	5	1	0
	6	6	0	0	3	3	0
Total		404	132	8	107	141	16

+ ; positive +? ; faintly positive ± ; ambiguous
? ; uncertain − ; negative

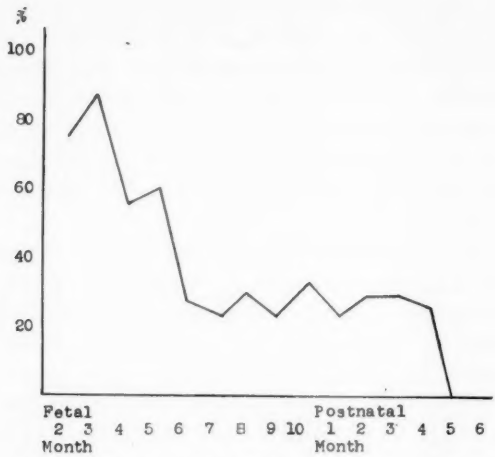


Fig. 1. Incidence of positive stain by the month.

TABLE II. Sex Incidence of Postitive Stain in Monthly Distribution

Age		Total		Male		Female		Unknown	
		Case	+	Case	+	Case	+	Case	+
Fetal Month	2	4	3	2	2	0	0	2	1
	3	28	24	19	15	6	6	3	3
	4	20	11	10	4	8	5	2	2
	5	27	16	15	9	11	6	1	1
	6	53	33	22	1	31	12		
	7	48	11	22	5	26	6		
	8	44	12	24	7	20	5		
	9	30	7	12	2	18	5		
	10	57	17	26	5	31	12		
Postnatal Month	1	46	10	23	4	23	6		
	2	16	4	10	4	6	0		
	3	12	3	5	1	7	2		
	4	7	1	4	1	3	0		
	5	6	0	4	0	2	0		
	6	6	0	3	0	3	0		
Total		404	132	201	60	195	65	8	7

was high in the first half of fetal period in general, became definitely lower in the second half and fell to zero after the 4th postnatal month, as is shown in the percentage curve of Fig. 1. The distribution of cases with positive reaction of each sex is represented in Table II in reference to month. The figures demonstrate that no significant sex difference was present in the first half of fetal period and in the postnatal period, but in the second half of fetal period, the female predominated significantly over the male in the rate of positive reaction.

To locate the site of cells with positive reaction, their distribution in the adrenal cortex was tentatively classified in the following four types.

Type A: The cells are diffusely distributed in the whole cortex.

Type B: The cells are distributed in the cortex, excluding the superficial layer just below the capsule, represented mainly by the glomerulosa.

Type C: The cells are distributed only in the fetal cortex.

Type D: The cells are only found in the remaining fetal cortex, after its considerable involution.

Type A had its highest incidence in the second and third fetal months, rapidly decreased its appearance in the following months and ceased to appear with the tenth fetal month. Type B comprised only a smalll number of cases, but had the similar tendency as Type A, inspite of some inconstancy. Type C began to be seen in the third fetal month, with a marked increase in frequency in the later period of pregnancy and disappeared in the first postnatal month. In this period Type C was replaced by Type D, which continued to be seen to the fourth month, including all positive cases since the second postnatal month (Table III). It was thus revealed, that in the relatively early embryonal stage

Table III. Distribution of Individual Type by the Monthly Distribution

Age / Type	Fetal Month									Postnatal Month						Total
	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	
A	2	19	4	5	3	2	1	2	0	0	0	0	0	0	0	38
B	1	1	3	3	1	0	1	0	0	0	0	0	0	0	0	10
C	0	4	4	8	9	9	10	5	17	9	0	0	0	0	0	75
D	0	0	0	0	0	0	0	0	0	1	4	3	1	0	0	9
Total	3	24	11	16	13	11	12	7	17	10	4	3	1	0	0	132

the cells containing ponceau-fuchsinophilic material were diffusely distributed in the whole adrenal cortex, with and without exception of the uppermost layer, but with the progress of fetal age they became gradually localized in the fetal cortex and disappeared with the accomplishment of involution of the fetal cortex. In a random sample of human adrenals from 7th postnatal month to the 64th year of age, the ponceau-fuchsinophilic granules were nagative except in a single case. The case was that of a 17 years old male, who had compensatory hypertrophy of the preserved adrenal, due to complete unilateral destruction of the adrenal by a huge mass of hypernephroma. The granules were found in the deep layer of the preserved organ.

Morphologically the granule containing cells were characterized by diffuse or coarse granular intracytoplasmic substances with characteristic bright red colour of ponceau-fuchsin. They resembled the so-called dark cells in morphological

properties, inspite of some variation in size according to the site of appearance.

The next step of the investigation was to detect ponceau-fuchsin granules in the course of development of the adrenal cortex, by means of serial sections prepared from 23 cases of human embryos from 4 mm to 24 mm in length. It has been acknowledged by many investigators, that the adrenal cortex originates from coelomic wall at the time when the embryo reaches the length of 7–8 mm. But in the present study the author could confirm already in 5 mm long embryos increased cell density with mitotic cell proliferation in the part of coelomic wall, which represented the origin of later adrenal cortex. Mitosis was found not only in coelomic lining cells, but also in adjacent mesenchyma. In 6 mm long embryos the cell mass of coelomic wall was more distinctly identified in the surrounding mesenchymal tissue as a continuous proliferation of coelomic epithelium. At the time when embryos became 7 mm in length, the cell mass could definitely be recognized as the origin of adrenal cortex. As the fetal month advanced, the cell mass became gradually larger with constituent cells similar to those of later permanent cortex. The cells were small and their protoplasm was not stainable with eosin or fuchsin. When embryos exceeded the length of 9 mm, the protoplasm of these cells obtained slight affinity to eosin, and scattered large nuclei began to be seen. In over 10 mm long embryos the cell mass was distinctly demarcated from the surrounding tissue with a gradually developing capsule and capillaries extending in it. In embryos over 15 mm in length well developed capillaries divided the cell mass into distinctive cell cords. The cells, which were situated in central parts of the cell cords, became rich in protoplasm. Dark and light cells could be discriminated and reticular arrangement of cells were estabished. Ponceau-fuchsin positive granules could not yet be demonstrated. At the fetal length of 20 mm, histological structures as the adrenal cortex were clearly recognizable, and outer and inner zones were differentiated. From this period on, cells with ponceau-fuchsin positive granules began to appear, scattered among the cells of inner layer with abundant protoplasm. At the same time siderophil and fuchsinophil granules could likewise be demonstrated by other staining methods. With the advancement of fetal development, ponceau-fuchsin granules became progressively conspicuous, together with the increase of granule containing cells.

DISCUSSION

In numerous works concerning the histogenesis of the adrenal cortex, there is a general agreement, that the adrenal cortex has its origin in coelomic wall of embryos of 7–8 mm in length. The investigation of the author could likewise confirm the cell mass representing adrenal origin in this stage of development. However, the author could find increased density of cells in this part of coelomic wall with mitotic cell proliferation already in embryos of about 5 mm. Of the

previous works the view of Gruenwald[10] is supported by this finding, that early cortex primordium is the part of mesenchyma differentiated from dividing cells of coelomic lining epithelium. The author can not agree with Uotila[17] and others, when they state that the fetal cortex is formed at first and at the stage of 12 mm fetal length the permanent cortex independently develops from coelomic epithelium. The author's own finding did not suggest a new additional element from coelomic epithelium since the stage of 12 mm fetal length, though the cells in central parts of the above-mentioned cell mass were slightly enlarged at that time. The formation of epithelial cords was established with 15 mm fetal length, and ponceau-fuchsin granules, specific for fetal cortex, appear only after embryos exceeded the length of 20 mm at the stage where the cell arrangement of the adrenal cortex in fetal age was accomplished with distinctive cell cords composed of the outer permanent cortex and the inner fetal cortex.

The cells with ponceau-fuchsin granules, which were at first distributed in both layers of adrenal cortex, fetal and permanent, generally became localized in the fetal cortex with the advancement of fetal period and totally disappeared with the maturity of the embryo.

It must be pointed out at the same time, that ponceau-fuchsin granules often failed to be demonstrated. It was indeed difficult to obtain specimen of human organs under uniform conditions, because of varying postmortem time and other factors, but the results were sometimes negative, inspite of strictly controlled conditions. As is shown in Fig. 2, the rate of positive incidence of the granules was highest in the third fetal month, moderately fell in the 4th and 5th months, became remarkably lower since the 6th month and fell to zero after the 5th postnatal month with the disappearance of the fetal cortex. This tendency was common in both sexes and without any significant sex difference, in which the author's result was different from the description of Vines. As pointed out in the introduction, it is a prevailing view today, that the fetal cortex is maintained by chorionic gonadotropin (LH), as it is the case with the X-zone of mouse. The excretion of the hormone in urine of pregnant women is regarded to be high in the earlier period of pregnancy in general and highest around the 8th to 12th weeks of pregnancy. Bruner[6] reported that the hormone content of fetal tissues was highest around the 11th to 12th weeks of pregnancy, rapidly decreased thereafter and, at the end of pregnancy, fell to about 1/25 of the highest value. This fluctuation is similar to that in the curve given in Fig. 2. Zinsser and Zinsser[20] regarded ponceau-fuchsinophil substances as ketosteroidprotein complex. At least it is clear that ponceau-fuchsinophil substances indicate the presence and activity of cells belonging to the fetal cortex. It is noteworthy, therefore, that the cells containing ponceau-fuchsin granules come to be seen only after the fundamental structures of adrenal cortex is established at the fetal length of 20 mm, show a similar fluctuation to that of chorionic gonadotropin content in fetal

tissues and change their earlier distribution in the whole cortex including the superficial layer, to be restricted in the fetal cortex in the later fetal period.

CONCLUSION

In the cortex of fetal adrenals the ponceau-fuchsinophil granules of Vines were demonstrated only after the establishment of fundamental structures of adrenal cortex, at the fetal length of 20 mm. No sex difference was confirmed in the incidence rate of the granule containing cells. The rate of positive incidence was highest in the second and third fetal months, fell gradually with the advancement of pregnancy and became very low in the later half of fetal period. The granule containing cells were generally distributed in the whole cortical layers in the earlier fetal period, but became localized in the fetal cortex in the later fetal period.

Acknowledgement : The author's study is initiated under the direction of the former Professor Dr. S. Nasu.

References

1) Bachmann, R., Die Nebenniere. Möllendorff's Handbuch der mikroskopischen Anatomie des Menschen. **VI**/5, Springer, Berlin, 1954.
2) Bernirschke, K. *et al.*, Endocrinology, 1956, **58**, 598.
3) Blackman, S.S., Bull. Johns Hopkins Hosp., 1946, **78**, 180.
4) Botella-Llusiá, J., Arch. Gynäk., 1953, **183**, 73.
5) Brow, H., Biological Action of Sex Hormones. Cambridge, 1949.
6) Bruner, J-A., J. Clin. Endocrinol., 1951, **11**, 360.
7) Carnes, W.H., Proc. Soc. exp. Biol. & Med., 1940, **45**, 502.
8) Gersh, I. & Grollman, A., Anat. Rec., 1939, **75**, 131,
9) Giroud, C.J.P. *et al.*, In Vitro Studies of the Functional Zonation of the Adrenal Cortex and of the Production of Aldosterone. An International Symposium on Aldosterone. p. 56, J. & A. Churchhill, London, 1958.
10) Gruenwald, P., Anat. Rec., 1946, **95**, 391.
11) Jones, I.C., The Adrenal Cortex. Cambridge, 1957.
12) Lanman, J.T., Medicine, 1953, **32**, 389.
13) Neumann, H.O., Arch. Gynäk., 1936, **162**, 289.
14) Race, G.J. *et al.*, Arch. Path., 1957, **64**, 312.
15) Rotter, W., Virchows Arch., 1949, **316**, 590.
16) Selye, H. & Stone, H., On the Experimental Morphology of the Adrenal Cortex. Charles C Thomas, Springfield, 1950.
17) Uotile, U.U., Anat. Rec., 1940, **76**, 183.
18) Vines, H.W.C., The Adrenogenital Syndrome. Some Histological Observations; in Broster *et al*'s The Adrenal Cortex and Intersexuality. 2nd ed., p. 137, Chapmans Hall, London, 1938.
19) Verzár, F., Die Funktion der Nebennierenrinde. Benno & Schwabe, Basel, 1939.
20) Zinsser, A.D. & Zinsser, H.H., Arch. Path., 1951 **51**, 393.

Fig. 2. Dense aggregation of cells of the excavation of coelomic wall which gives rise to the origin of the adrenal cortex. A horizontal section of an embryo 6 mm in length.

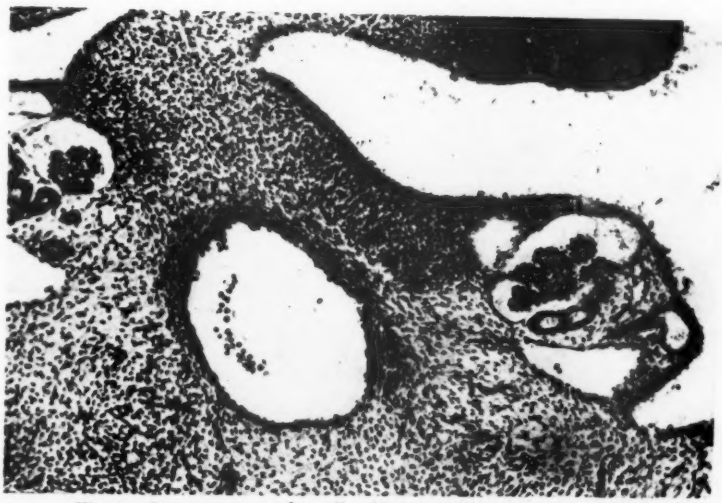


Fig. 3. Dense masses of small cells by proliferation of epithelial and mesenchymal cells of the coelomic wall. It becomes evident that these cell masses are an origin of the adrenal glands. A horizontal section of an embryo 8mm in length.

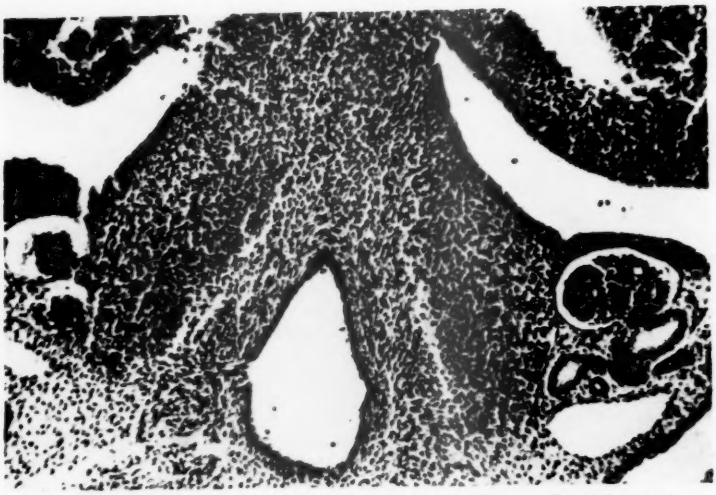


Fig. 4. The central cells in the cell masses enlarge and yet the protoplasm does not exhibit the characteristics of the cortical cells. A horizontal section of an embryo 9.5 mm in length.

Fig. 5. The adrenals show further development. The cytoplasm of the inner cortical cells becomes evident and the structure of the adrenal cortex and the capsule are to be made. Ponceau-fuchsinophilic granules become observable from this stage. A horizontal section of an embryo 23 mm in length.

Tohoku J. Exper. Med., 1961, 73, 127–146

Studies on X-Agent

VI. Effect of X-Agent on Red Blood Cells

By

Hideo Moriyama

Shonan Hygiene Institute, Kamakura, Japan

(Received for publication, August 29, 1960)

Evidence has been presented that there is an unidentified physical agent capable of exerting striking effects upon the growth of bacteria and plant seeds[1][2][3]. It has been confirmed that this agent has also influence upon protein solutions to cause a change in the turbidity[4]. It is considered that the turbidity change may result from a structural activation or excitation in the protein molecule. Since all the organisms are composed of proteins, this agent having thus an influence upon the protein must have effect on all the living things.

It is my object in this paper to communicate the fact that the effect of the X-agent can be demonstrated likewise in red cells which are a kind of free cells of ourselves.

Red cells have been adopted as an experimental object, because a red cell liberates hemoglobin in a quantity which is directly proportional to the injured degree of its protoplasm,[5] so that the measurement of the liberated hemoglobin amount enables one to estimate the injured degree of the cell. As will be mentioned in another paper, the change of protein solution due to the X-agent is apt to be reversible, so that experimental results obtained using protein solutions have to be scrutinized with much prudence. On the other hand, the change induced in red cells will cause the liberation of hemoglobin, and since the hemogloben liberation is irreversible, one can easily estimate the degree of the change occurring in the cell by measuring the amount of liberated hemoglobin without fearing of the reversibility.

X-agent itself is unable to induce hemolysis or the liberation of hemoglobin, only causing a change in the cell or only capable of determining the structural feature of the cell-protoplasm. However, if some adequate hemolytic agent such as a hypotonic salt solution is applied to such a cell in a concentration to cause a partial hemolysis, hemolysis will occur or hemoglobin will be liberated in an amount which is regarded as being dependent upon the degree of the change, or dependent upon the structural feature of the cell.

守 山 英 雄

MATERIALS AND METHODS

Human red blood cells were used throughout the experiments. The blood cells were isolated by centrifugation from citrated blood, washed three times with 0.9 per cent NaCl solution, and suspended in the saline in an amount of about 2 per cent. 1.0 ml of this suspension contained in a test tube was placed under a given condition of the X-agent, and after being left for a certain period of time 5.0 ml of a hypotonic NaCl solution was mixed, and again left for 10 minutes under the same condition. In most cases 0.35 per cent NaCl solution was used as the hypotonic solution, for it seemed to be suited to cause a partial hemolysis. 10 minutes were enough for the hemolysis to be completed whose degree was directly proportional to the degree of the change induced by the hypotonic solution and the X-agent. After the completion of hemolysis whose degree was dependent upon the situation of the red cells, the cells were centrifuged as quickly as possible and the hemoglobin amount contained in the supernatant was measured by the Leitz-photometer using the filter No. 550. All the test tubes used in the experiments had been made by cutting about 9 cm in length, so that the contents of the tubes could be centrifuged without being transferred to the ordinary centrifuging tubes; the test tubes themselves could be used as centrifuging tubes.

Instead of the hypotonic solution, $HgCl_2$ was employed in some experiments. In such a case, $HgCl_2$ was dissolved in 0.9 per cent NaCl solution at the concentration of M/1,000, and its 5.0 ml was mixed with 1.0 ml of the red cell suspension. 2 hours after the mixture the red cells were separated by centrifugation from the liberated hemoglobin, which was measured as stated above.

RESULTS

In the case of using $HgCl_2$ as hemolytic agent

Firstly, results of experiment which was carried out using $HgCl_2$ as the hemolytic agent will be presented. All the results dealt with in this paper were acquired in 1959, excepting those shown in Fig. 6 which were obtained in 1960.

It was confirmed previously[5], that the red cells acted upon by $HgCl_2$ with an adequate concentration would undergo a partial hemolysis occurring very gradually, whose degree was dependent upon the injured degree of the red cells. It seemed that red cells whose structural feature was under the influence of the X-agent would liberate, by the action of $HgCl_2$, a hemogloben amount which is considered to be dependent upon its structural feature, so that from the amount of liberated hemoglobin one can estimate the degree of influence of the X-agent.

The pattern of influence of water bottle upon the red cells placed around it was obtained in the same way as in the previous investigations performed using the bacteria[2], or the plant seeds[3], or the protein solution[4].

16 test tubes each containing 1.0 ml of the red-cell suspension were arranged

in double circles around a glass bottle containing 1 liter of water (see Fig. 1). 5.0 ml of $HgCl_2$ solution was added to each test tube thus arranged, and after mixed well, left at room temperature for 2 hours, after which red cells were separated by centrifugation, and the hemoglobin amount in the supernatant was measured. The results are shown in Fig. 2.

Solid and broken curves in this figure represent the values obtained with the red cells contained in test tubes arranged respectively in the inner circle and

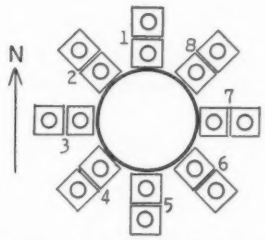


Fig. 1. 16 test tubes of red cells are arranged in two circles around a glass bottle containing 1 liter of water. In this manner patterns presented in Figs. 1 and 2 were obtained. Each tube was placed in a small box.

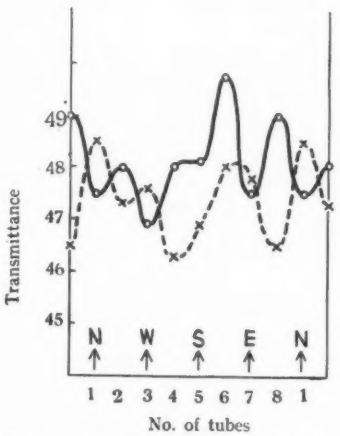


Fig.2. Patterns around a water bottle. $HgCl_2$ was used as the hemolytic agent. Solid and broken curves represent the pattern in the inner circle and that in the outer circle respectively. When water was added instead of $HgCl_2$ solution, the transmittance of the supernatant was found to be 34.2, that is, the transmittance in the case of complete homolysis was 34.2.

in the outer circle. Effect of the water bottle thus varied with directions.

It should be noted that the two curves obtained respectively in the inner circle and in the outer circle are not parallel, but tending to be mirror-imaging one another.

In the case of using the hypotonic solution as the hemolytic agent

A hypotonic NaCl solution is much more suited than $HgCl_2$ solution for the purpose of investigation, since the hemolysis by the hypotonic solution will be completed in a few minutes unlike that caused by $HgCl_2$ solution which as a rule may require an extremely long time for its completion. For this reason, a hypotonic NaCl solution was used in almost all the investigations of which mentions will be made hereafter; there seemed no essential difference in the results attributable to the hemolytic agent used.

The curves shown in Fig. 3 were obtained in essentially the same manner as in Fig. 2. Thus, test tubes containing 1.0 ml of red-cell suspension were arranged in double circles around a glass bottle containing water. 30 minutes after the arrangement, 5.0 ml of 0.38 per cent NaCl solution was added to each test tube and left under the same condition, after well mixed, for further 10 minutes, and

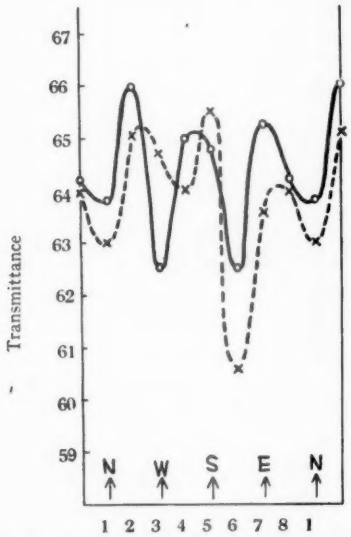


Fig. 3. Patterns around a water bottle. A hypotonic NaCl solution was used as the hemolytic agent. Solid and broken curves represent the pattern in the inner circle and that in the outer circle respectively.
The transmittance at complete hemolysis was 35.5.

then the liberated hemoglobin amount was measured, after the red cells being separated by centrifugation. The resemblance of the two curves is seen in Fig. 3.

When experiments were made with test tubes arranged in a manner shown in Fig. 4, the resemblance between two patterns obtained with two groups of tubes

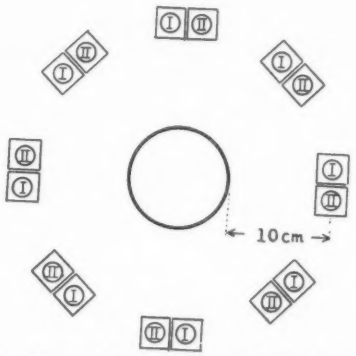


Fig. 4. 16 test tubes are arranged in a large circle, each two tubes making a group. In this manner patterns shown in Fig. 5 were obtained.

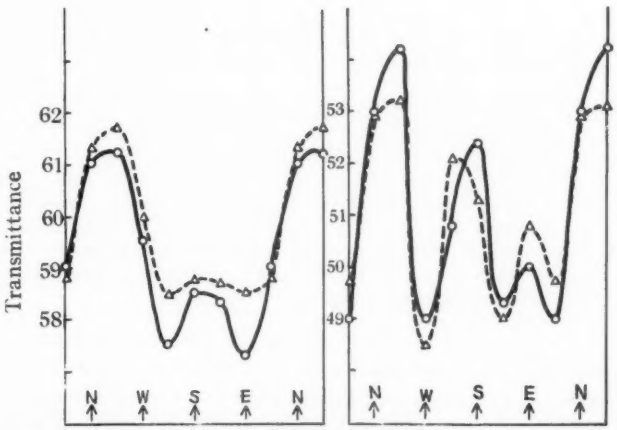


Fig. 5. Patterns around a water bottle. Solid and broken curves represent the patterns attained respectively with tubes I and II shown in Fig. 4. The two patterns were obtained on different days in Dec. 1959.

became generally more manifest as seen in Fig. 5*.

Similar patterns could be demonstrated with bacteria and proteins not only around a water bottle but also around many other material substances[2)4)]. This was likewise confirmed with red blood cells as shown later in this paper. This can never be ascribed to a known physical factor such as temperature and light, a conclusion which was already attained and which can be readily reached from the experimental results shown in Fig. 6. In this figure, three patterns obtained around each similar water bottle placed at different temperatures are presented. The temperatures at which they were acquired were as follows. I: 35 C; II: 20 C; III: 9 C.

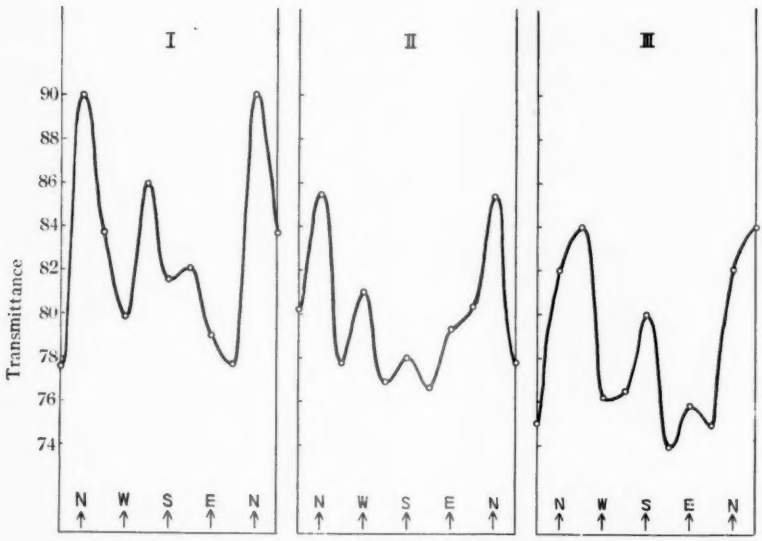


Fig. 6. Patterns around water bottles placed at different temperatures. Curve I was obtained in an incubator of 35 C using 8 test tubes arranged closely around a glass bottle containing 1 liter of water. Curve II was acquired in the same manner in an incubator of 20 C. Curve III was found around a water bottle placed on a table at laboratory temperature (9 C).

Date of experiment : Feb. 11, 1960.

* The two curves presented in this figure tend to be identical or parallel. This is naturally expected since every two tubes were located side by side so that the X-agent would have a similar influence on the two tubes. However, two curves obtained in a similar manner were not always identical or parallel, but frequently one curve tended to be the mirror image of the other, as was the two curves shown in Fig. 2. Regarding this curious phenomenon a detailed mention will be made in a forthcoming paper.

It is shown in this figure that hemolysis tended to occur more highly as the temperature was lower, but the difference in the hemolysis grade due to the temperature difference, even so great as covering nearly 30 degrees, is rather insignificant as compared with that due to the place difference around a water bottle. There was no temperature difference around a water bottle detectable by an ordinary thermometer. Even if some detectable temperature difference could exist around a water bottle, the pattern could not be attributed to it.

The visible light can likewise be readily excluded, because both patterns I and II were attained in incubators shut out from the outer source of light while III was obtained on a table in the laboratory open to the outer light. There is no essential difference in the patterns between these situations.

As repeatedly discussed, the factor involved in this phenomenon is supposed to be the X-agent having a very strong power of penetration, and the pattern observed around a material substance, such as a water bottle, is believed to result mainly from a secondary agent or radiation emitted from the substance upon which the primary agent is coming[2].

Difference in the pattern due to the day of investigation

As stated in the previous papers, essential feature of the X-agent consists in its variability with both time and space, that is, the X-agent varies remarkably with time and space. This can likewise be finely demonstrated with red cells.

In Figs. 7 and 8 is shown how the pattern of the X-agent is variable with time. Curves in the figures are obtained with a group of test tubes containing red blood cells which were arranged around a glass bottle. Curves shown in the upper part of the figures were acquired with an empty glass bottle, while those in the lower part with a glass bottle containing 1 liter of water. Each bottle was placed always in a given place on the same table, so that there could be no difference due to the place so far as the same bottle was concerned. In the experiments shown in Figs. 2 and 3, the distance between the surface of the bottle and the center of test tube placed around the bottle was 1.5 cm, while in these cases it was 7.5 cm, that is, in the former cases the test tubes were placed very close to the surface of the bottle, while in these cases they were arranged far from the surface in a much larger circle.

Curves shown in the upper part of Fig. 7 are much different from those in the lower part. Since the empty bottle, which gave the upper curves, and the water bottle, which provided the lower curves, were placed in different places, there must be a difference in the pattern due to the place difference. However, it is reasonable to consider that the great difference found between the upper and the lower curves is in the main attributable to the difference in the bottles around which the test tubes were placed; as just mentioned, the upper curves were obtained with an empty bottle while the lower curves with a bottle con-

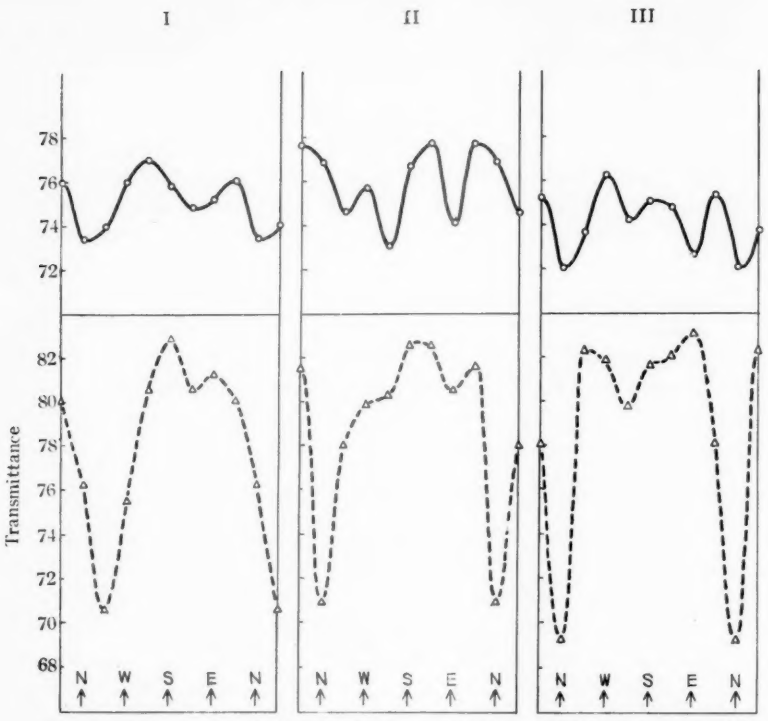


Fig. 7. Patterns around glass bottles, one of which was filled with water while the other was empty.

Solid curves : empty bottle; broken curves : bottle with water.

Date of observation; I : Apr. 20; II : Apr. 21(A.M.); III : Apr. 21 (P.M.), 1959.

taining water.

Such a difference, however, was not always observable as seen in Fig. 8 in which curves obtained on the other days are presented. The curves in the figure fail to show any difference attributable to the existence or non-existence of water in the bottle.

In such a way, the pattern of X-agent varied strikingly with days of observation. Curves were found to be similar on both April 20 and 21 as shown in Fig. 7, but after 2 days, that is, on April 23 a marked change occurred in the curves; a further change, though a little, was demonstrated 4 days after, *i.e.*, on April 27 (see Fig. 8).

It is worthy of note that the curves obtained with the water bottle as shown

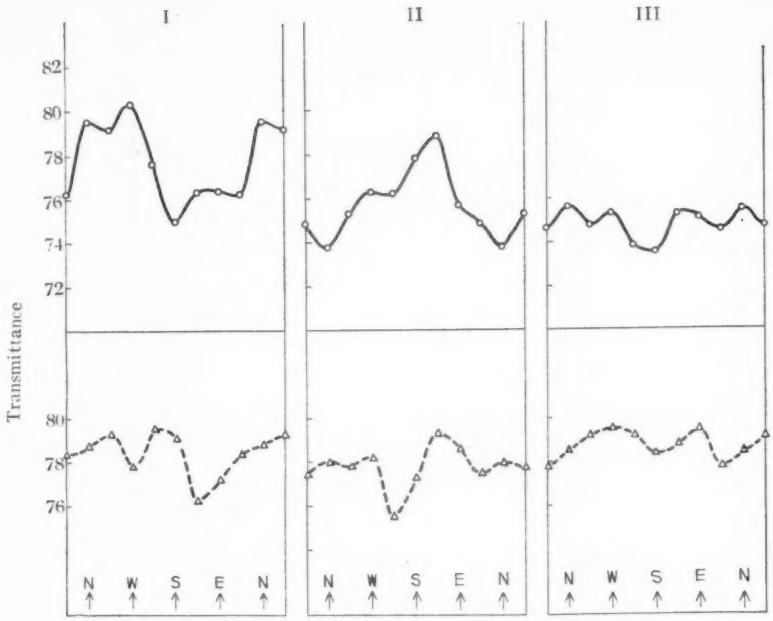


Fig. 8. Patterns around glass bottles, one of which was filled with water while the other was empty.
Solid curves : empty bottle; broken curves : bottle with water.
Date of observation; I : Apr. 23 (A.M.); II : Apr. 23 (P.M.); III : Apr. 27 (A.M.), 1959.

in the lower part in Fig. 7 exhibit a very deep valley in the north direction. As discussed earlier[6], the X-agent seems to progress frequently along the direction extending north and south. The development of such a valley can be accounted for by the effect of the direction along which the X-agent was running on the experimental days. This valley, however, was completely lost after 2 days, suggesting the occurrence of a change either in the direction of the progress or in some other characters of the X-agent.

Patterns around cans either empty or filled with water

The phenomena described above, of course, were never peculiar to glass bottles. In the following a mention will be made regarding experimental results obtained with cans, whose diameter and the content were respectively 6.5 cm and 300 ml.

Observations were made with two given different places (I and II) on a table. Distance between I and II was 50 cm, II being north of I. Two cans one

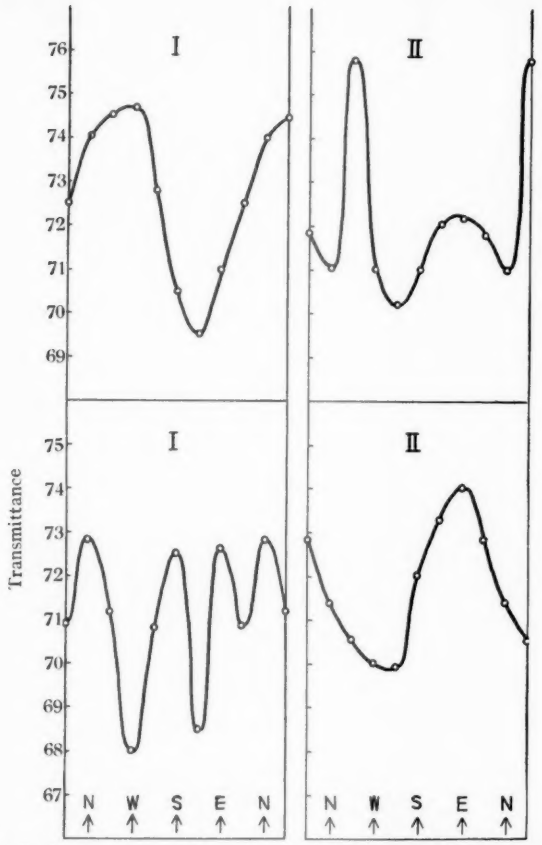


Fig. 9. Patterns around cans, one of which was filled with water while the other empty.

Upper curves were obtained in the morning of April 7; I with a can filled with water, II with an empty can.

Lower curves were obtained in the afternoon of April 7; I with the empty can; II with the can filled with water.

of which was empty and the other filled with 300 ml of water were placed in these two places, and the patterns around these two cans were investigated at the same time. The two curves shown in the upper part in Fig. 9 were attained in the morning of April 7; curve I was obtained with water can which was placed in place I and curve II was of an empty can placed in place II. Curves in the lower part was obtained in the afternoon of the same day, curve I being of the empty can

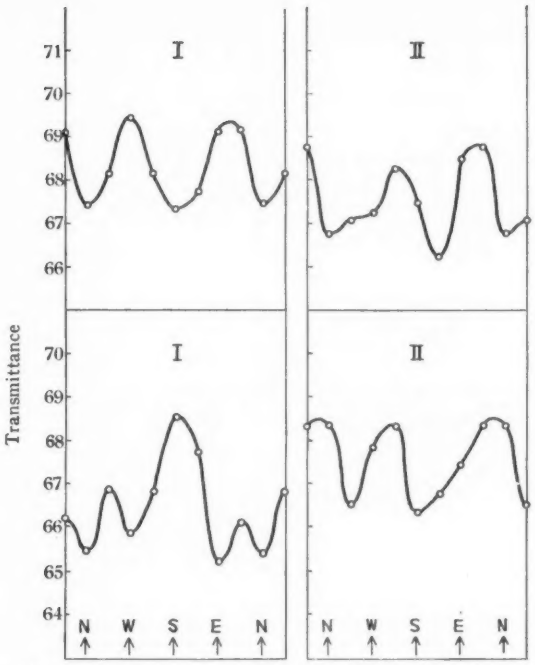


Fig. 10. Patterns around cans, one of which was filled with water while the other empty.

Upper curves were obtained in the morning of April 8; I with a can filled with water, II with an empty can.

Lower curves were attained one hour after the upper curves were obtained in the morning of April 8; I with the empty can, II with the wather can.

placed in place I, while curve II of the water can placed in place II.

There seems to be a slight difference between the empty and water can, the latter tending to give patterns more smooth than those of the empty can regardless of the difference in places. But such a difference could not be demonstrated on another experimental day as shown in Fig. 10. Namely, patterns are similar irrespective of the presence or absence of water in the can. In a similar way, as stated above, difference between the water bottle and the empty bottle could be demonstrated only on some experimental days.

It may safely be concluded, therefore, that some difference is revealed between empty and water vessel at least on certain days. However, the difference is only a little as compared with that due to the experimental days.

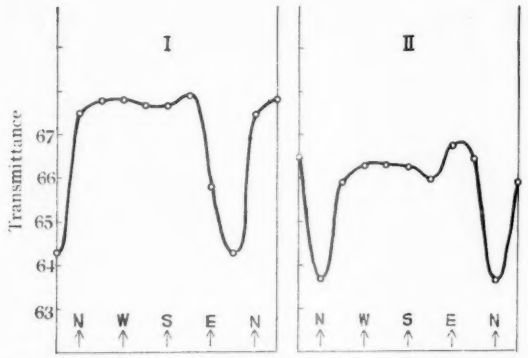


Fig. 11. Patterns around two cans, one of which (I) was empty while the other (II) filled with water.

The investigation was made at the same time in different places on April 8.

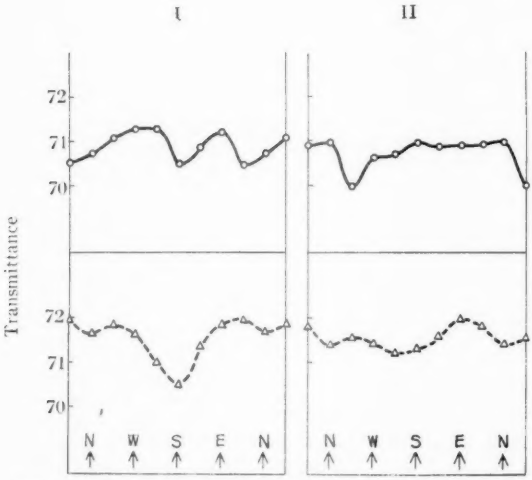


Fig. 12. Patterns around two cans, one of which was empty while the other filled with water.

The cans were placed in the same place; but the investigations were made at different times in the morning of April 23.

Solid curves were obtained with water can, while broken curves with empty can.

Time of investigations; solid curve (I) : 8–9 o'clock; broken curve (I) : 9–10 o'clock; solid curve (II) : 10–11 o'clock; broken curve (II) : 11–12 o'clock.

Thus, as shown in Fig. 11, the patterns obtained on April 8 are very characteristic exhibiting a deep valley in north or north-east direction as in the case of water bottle on April 20 and 21 (see Fig. 7). This may be attributed to a similar, peculiar character of the X-agent on these experimental days. With glass bottles the deep valley was seen only with the water bottle, while with the cans, the valley was observed regardless of the presence or absence of water in the can. It is quite unknown whether this was attributable to the difference in the vessel or to the different character of X-agent itself.

At any rate, it is certain that the agent exhibits from time to time very characteristic feature which may be maintained for one or two days and then lost.

Experiments whose results are presented in Figs. 12 and 13 were carried out in succession alternatively with water and empty can which were placed in the same place on the same table. It is remarkable that no identical patterns could be obtained even when the observations were made with the same substance and with the same place, but with the time a little different.

All the curves in Fig. 12 are very flat, and no differences are demonstrable

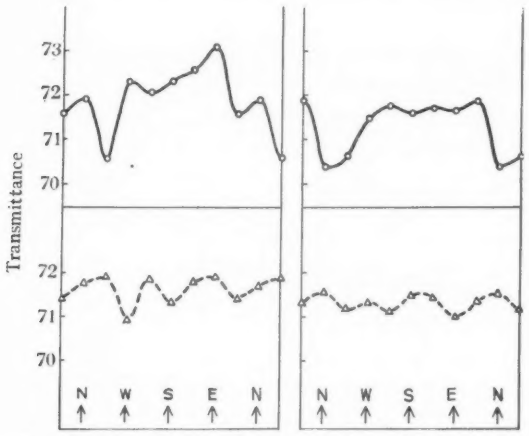


Fig. 13. Patterns around two cans, one of which was empty while the other filled with water.

The cans were placed in the same place, but investigations were made at different times.

Solid curves were obtained with water can, while broken curves with empty can.

Time of investigations; solid curve (I) : 13–14 o'clock on April 23; broken curve (I) : 14–15 o'clock on April 23; solid curve (II) : 9–10 o'clock on April 25; broken curve (II) : 10–11 o'clock on April 25.

between the water can and empty can. Curves shown in Fig. 13 are likewise flat but a difference between the two cans is demonstrable, the water cans tending to present the curves like those in Fig. 11, exhibiting valley in the north direction.

Thus it is evident that the pattern of X-agent varies greatly with the day of observation; the variation is generally much greater than that due to the difference in material with which the observation is made.

Difference in the pattern due to the place of the observation

The patterns obtained with a certain material substance at the same time are as a rule not identical when they are obtained in the different places even though the observations are performed on one and the same table. This is the case not only with the places different in horizontal but also with those different in perpendicular as shown in Fig. 14.

In this experiment the material placed in the center of the circle of the test tubes was a can filled with 300 ml of water. The difference in space either in horizontal or in perpendicular thus resulted in the difference in the pattern.

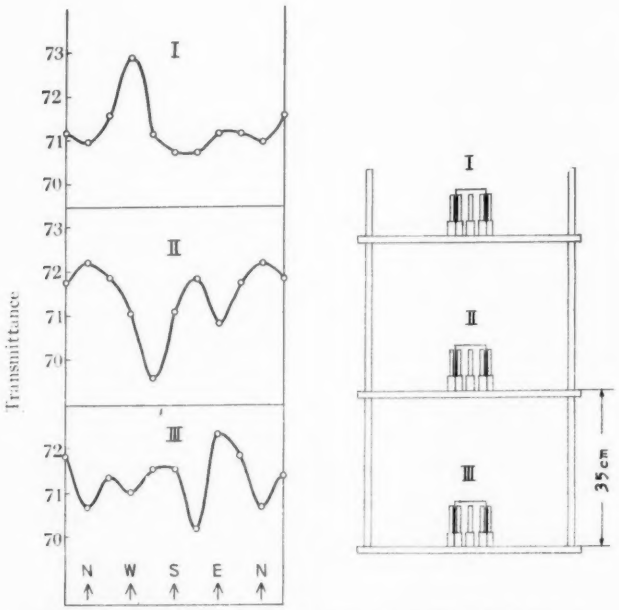


Fig. 14. Patterns around three water cans placed perpendicularly different places.
Date of investigation : April 13.

This is naturally expected since the distance between the can and the floor or the earth from which the secondary radiation might be coming was not the same among these three cans.

Difference in the pattern due to the direction of the line in which the test tubes were arranged

The difference in place is manifest even when the test tubes are arranged in a line ; each test tube in a line reveals a different feature, and the pattern of such different features varies greatly with the direction of the line as previously communicated[6].

This could be demonstrated likewise with red cells as indicated in Figs. 15 and 16. The experiments illustrated in Fig. 15 were carried out with two groups of test tubes each of which was put in a small card-board box, one group being arranged in a line extending north and south and the other east and west, mutual interval of test tubes being 3 cm.

1.0 ml of the red-cell suspension was put in a test tube and left for 20 or 30 minutes at a certain position in a certain line, and then 5.0 ml of the hypotonic salt solution was mixed to induce hemolysis, 10 minutes after mixing the red cells were separated by centrifugation and liberated hemoglobin amount was estimated.

Zigzag pattern and gradually sloping pattern were obtained as in the case of protein solutions. According to the author's view, such patterns are produced chiefly by the X-agent which is progressing along the direction of the line. In the experiments illustrated in Fig. 16, test tubes were arranged in ditches made with lead blocks in a manner indicated in Fig. 17, in order to prevent as possible the interference of the X-agent which may run along directions other than that of the line in question. The ditch was provided with walls each of which was made with lead blocks having a thickness of 10 cm. With the same ditches analogous experiments were already carried out with protein solutions[6].

A distinguished pattern observed in a ditch is believed to be caused in the main by the X-agent running along the direction of the ditch. Therefore, it is considered that almost no X-agent was running along the direction of the ditch in which no or insignificant pattern was demonstrated. Thus it may be said that in the morning on September 2, almost no agent was progressing in II direction, while in the afternoon of the same day, no agent was running in IV direction. In the afternoon of the next day, that is, September 3, such directions were III and IV. It is worthy of note that the agent appeared to progress in most cases in the direction extending north and south, but the direction seemed to be changed, though a little, from time to time. The directions of ditch in which no patterns were observed varied presumably because of this little change of the direction. Patterns in I direction were always pronounced but the shapes of the

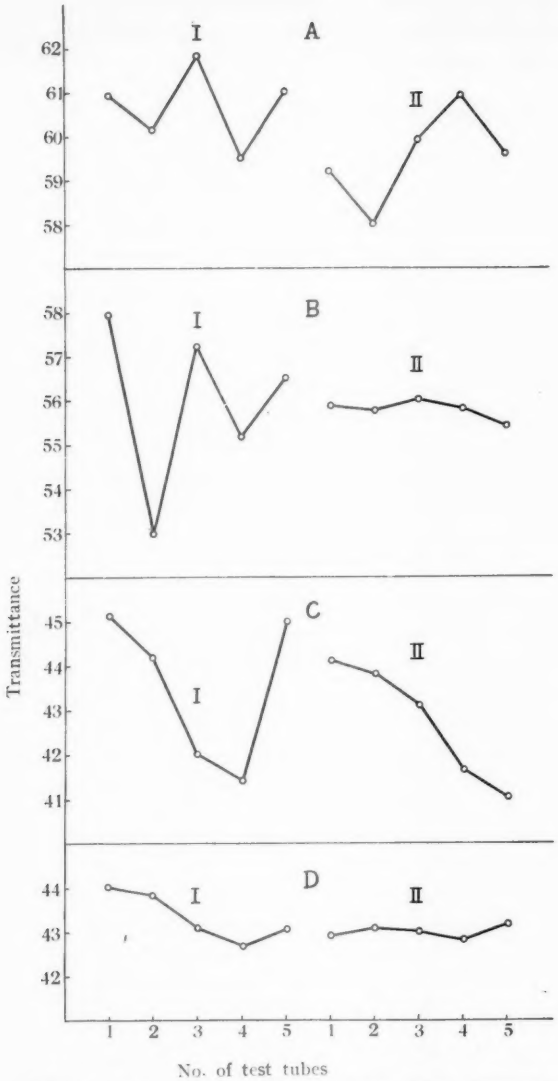


Fig. 15. Patterns of two groups of test tubes arranged in two lines, one of which extending south and north (I) and the other west and east (II).

Date of experiment; A : May 26; B : May 27; C : May 28 (A.M.); D : May 28 (P.M.).

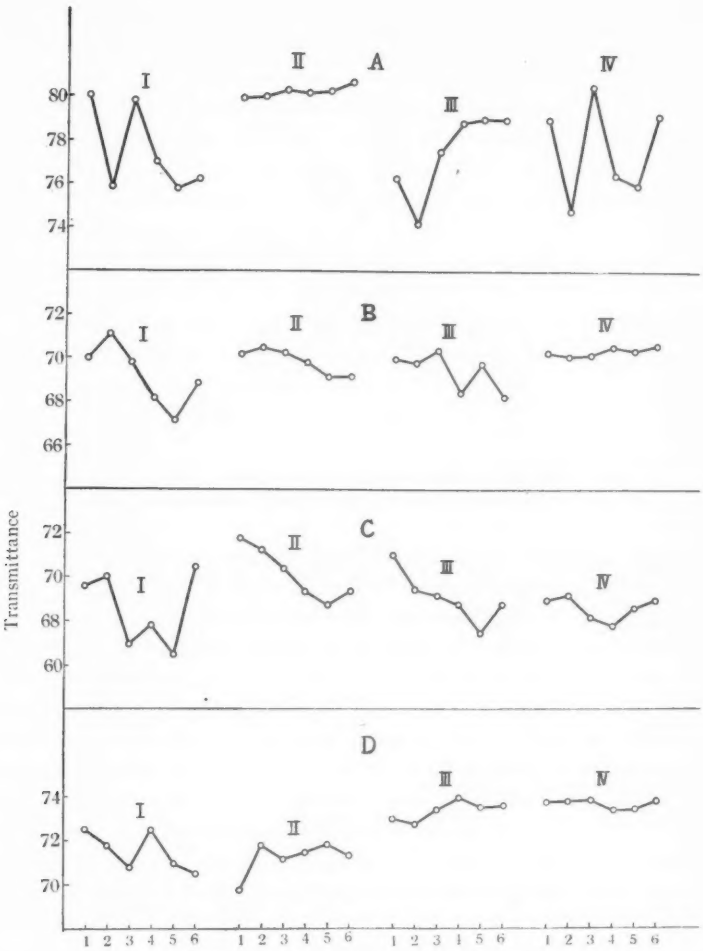


Fig. 16. Patterns of four groups of test tubes arranged in different ditches made with lead blocks. The manner of the arrangement is shown in Fig. 17.

Date of experiment; A : Sept. 2 (A.M.); B : Sept. 2 (P.M.); C : Sept. 3 (A.M.); D : Sept. 3 (P.M.).

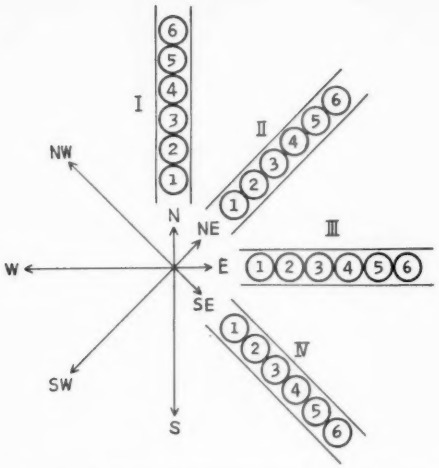


Fig. 17. Manner of arrangement of test tubes in four different ditches with so many different directions.

pattern were not identical, also suggesting the occurrence of such a little change.

To sum up, various phenomena which have previously been found with the bacteria and the protein could likewise be demonstrated with red blood cells. The change of the red cells was estimated by the amount of liberated hemoglobin, whereas, since hemoglobin is a kind of protein, if a protein is affected by the X-agent, hemoglobin solution itself will be changed by the agent, making the results extremely confused. In reality, however, no change was demonstrated in the hemoglobin solution which could be attributed to the effect of the X-agent. Hemoglobin amount was found always constant, apparently exerted no influence by the X-agent. Some property of hemoglobin itself might be changed by the agent, but, if such was the case, the color of hemoglobin would not be affected by the change of the protein moiety, so that the amount of hemoglobin was always found to be constant when the estimation was carried out on the basis of its color.

DISCUSSION

The various queer phenomena which were previously found with the bacteria and protein solutions have likewise been demonstrated with red blood cells. If the X-agent can have effect upon some structure in the protein molecule as considered previously[4], it will be only natural that the blood cells which are composed of proteins are influenced by the factor. It seems worthy of note, however, that it has actually been demonstrated that the cells of our own also are under the influence of the X-agent.

The X-agent itself has not any hemolytic action but appears to exert influence upon the feature of the protoplasm of red cells, so that the red cells will liberate hemoglobin amount dependent upon the feature of the protoplasm if acted upon by an adequate hemolytic agent. The X-agent of some characters may promote the hemolysis, while of other characters may on the contrary inhibit it. It should be pointed out that when the pattern of the X-agent was very characteristic as in the case of water bottles in Fig. 7, a deep valley was seen in the north direction, that is, a marked hemolysis took place in the north direction. This fact suggests that the X-agent of some characters which was running along the direction extending north and south tended to promote the hemolysis. It is remarkable, however, that sometimes, as seen in Figs. 5 and 6, a steep peak instead of a deep valley was observed in the north direction. In such a case, the X-agent running along the direction inhibits the hemolysis, supporting the just mentioned postulation that the agent may sometimes promote while sometimes inhibit the hemolysis depending on its character. At any rate, it is noteworthy that frequently a deep valley or a steep peak was observed in the north direction.

It also merits attention that the X-agent showed very striking changes from time to time in addition to a mild change occurring almost day after day. This striking change seemed to occur suddenly and continued for a few days and then lost. This may be called a storm of the X-agent, which was observed previously with both the bacteria and the protein solutions, and as reported in this paper also with the blood cells as seen in the example in Fig. 7. In such a storm, X-agent of a peculiar character seemed to be progressing in the direction extending north and south, leaving a pronounced pattern in the direction.

It should be expected that some characteristic changes in living organisms including human beings may be temporarily brought about by such a storm of the X-agent.

SUMMARY

Various phenomena, apparently concerned with the X-agent, which were previously found with the bacteria and the protein solutions have been demonstrated with human red blood cells.

It is believed that red cells, when mixed with an adequate hemolytic agent, such as hypotonic salt solution, will liberate a quantity of hemoglobin corresponding to the degree of change induced in the cells by the X-agent, so that the effect of the X-agent can be estimated by the measurement of the liberated hemoglobin amount.

Since the hemoglobin liberation or the hemolysis is irreversible, the effect of the X-agent could be estimated in this manner without fearing of reversibility of the change induced by the agent.

References

1) Moriyama, H. & Ueno, H., Japan. J. Microb., 1959, **3**, 387.
2) Moriyama, H. & Ueno, H., Japan. J. Microb., 1959, **3**, 411.
3) Moriyama, H., Japan. J. Microb., 1960, **4**, 7.
4) Moriyama, H., Japan. J. Microb., 1960, **4**, 83.
5) Moriyama, H., The Nature of Viruses and the Origin of Life. Igaku-shoin Co., Tokyo, Japan, 1955.
6) Moriyama, H., Japan. J. Microb., 1960, **4**, 229.

Tohoku J. Exper. Med., 1961, 73, 147-158

Studies on X-Agent

VII. Time-Fluctuation of X-Agent

By

Hideo Moriyama

Shonan Hygiene Institute, Kamakura, Japan

(Received for publication, August 29, 1960)

As reported in the previous paper[1], the effect of X-agent exhibited in a mere fragment of time can be estimated if investigations are made using red blood cells, since the red cells undergoing a change by the X-agent will liberate an amount of hemoglobin depending upon the degree of the change, and, as the hemoglobin liberation is irreversible, the estimation of the hemoglobin amount will reveal the degree of change induced in the cells.

Bacterial growth taken as a criterion of the study is not fitted for the purpose, as the growth established in a fragment of time may not be large enough to be measured. On the other hand, the turbidity of protein solutions may be changed according to the feature of the X-agent constantly changed[2]. The change in the turbidity, however, tends to be reversible, so that a change once established may be lost during the time of estimation of the turbidity because of the reversibility. Even when there is no such a reversibility, the turbidity will be changed during the short time of estimation, since the agent is acting upon the solution at any time and in everywhere.

For these reasons the studies on the feature of time-fluctuation of the X-agent seemed to be possible only when the red blood cells were employed. And actually, the author has been able to know, using the blood cells, of how the X-agent is changing constantly with time. The present paper is concerned with this finding.

MATERIALS AND METHODS

Human red blood cells were used. The blood cells separated from citrated blood by centrifugation were suspended at a concentration of about 2 per cent in a 0.9 per cent NaCl solution after washed three times with the saline. 1.0 ml of this suspension was mixed with 5.0 ml of a hypotonic solution of NaCl (in most cases 0.35 per cent solution was used) and 10 minutes after mixing the cells were centrifuged. The amount of hemoglobin present in the supernatant was estimated by the Leitz-photometer using the filter No. 550.

守 山 英 雄

In order to study the feature of constant variation of the X-agent, portions of the cell suspension which were placed under a given condition were removed at adequate time intervals, and mixed under a certain condition with the hypotonic solution to induce partial hemolysis which was dependent upon the degree of the change in the blood cells caused by the X-agent.

RESULTS

Experiments carried out using the red cell suspension contained in the same vessel

A red cell suspension contained in a beaker was placed at room temperature in a certain place in the laboratory, and at intervals of 10 minutes each 1.0 ml of the suspension was taken into every two test tubes each of which had been provided with 5.0 ml of the hypotonic salt solution. The test tubes whose length had been made 9 cm by cutting were used throughout the experiments, since such tubes can be used in themselves as centrifuging tubes.

The results are presented in Fig. 1. Two curves represent the results obtained respectively with every two test tubes placed about 10 cm apart on the same stand, one being placed in a position south to the other. The curve I and II were obtained with the test tubes placed respectively in the southern and the northern position. The disparity between these two curves is attributable to the disparity between the positions in which the two test tubes were plased. It has previously been communicated that, even in a group of test tubes standing in a line on a usual stand, each test tube reveals each different feature[1].

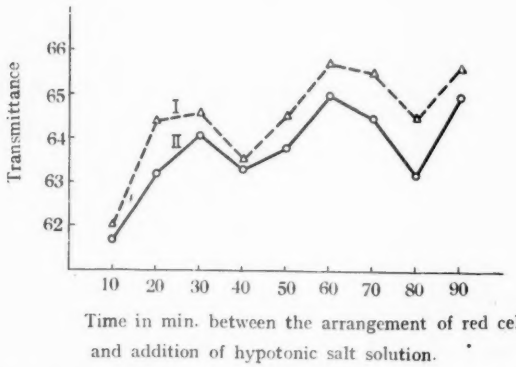


Fig. 1. Time variation of the amount of liberated hemoglobin.
Date of experiment : March 25, 1959.
I : Results obtained with I test tubes placed at southern position.
II : Results obtained with II test tubes placed at northern position.

It is remarkable that the amount of liberated hemoglobin varied with time, showing that the changing degree of blood cells was constantly being altered with time, an evidence that the X-agent was undergoing a constant time-fluctuation.

The blood cells contained in the same beaker must have been subjected to the same degree of change at the time of removing, but after the removing they were placed in different places where the hypotonic solution was mixed. Depending on this difference in places where hemolysis took place following the mixture of hypotonic solution, the disparity between two curves would result. It will be noted that, despite the presence of such a disparity, the two curves are quite similar in time-fluctuation, indicating that the presence of time-fluctuation in the X-agent is an undeniable fact.

Experiments carried out using red cell suspension contained in different vessels

A red cell suspension, if contained in two different vessels, will be exerted different influences by the X-agent in accordance with the different positions they are placed. And if a portion of the suspension is removed from each of these two vessels and submitted to a partial hemolysis in different test tubes by the addition of the hypotonic solution, they will further be exposed to the X-agent of different features, thus the difference in the degree of hemolysis becoming greater. However, since the X-agent itself which is exerting its influence both upon the different vessels and upon the different test tubes must be undergoing essentially a similar time-fluctuation, two curves obtained with a series of the two different tubes must reveal a similar feature.

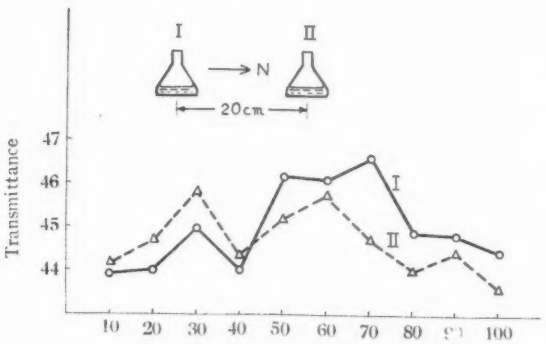


Fig. 2. Time variation of liberated hemoglobin amount.
Date of experiment : March 30, 1959.
I : Results obtained with red cells contained in I flask.
II : Results obtained with red cells contained in II flask.

For such a reason, two curves I and II shown in Fig. 2 may resemble to each other, although the difference is considerably great.

Curve I was attained with blood cells taken from the vessel I, and curve II with blood cells from the vessel II shown in the figure. At intervals of ten minutes 1.0 ml was removed from each vessel and mixed with 5.0 ml of the hypotonic salt solution contained in each test tube; the two test tubes in which the cells were submitted to hemolysis were placed in the same manner as in the above experiment.

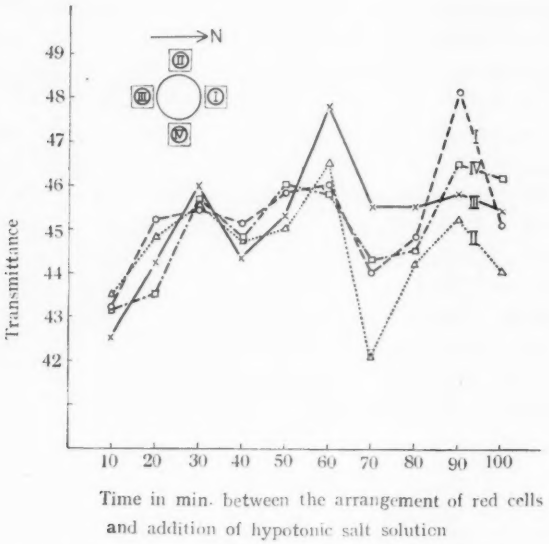


Fig. 3. Time variation of liberated hemoglobin amount.
Date of experiment : April 3, 1959.
Curves I, II, III, and IV were obtained with red cells contained respectively in glass vessels, I, II, III and IV, shown above.

In Fig. 3, the result of an experiment made with four different vessels was presented. The four vessels containing the red cells were placed around a glass bottle of 1 liter of water as indicated in the figure, and at intervals of 10 minutes 1.0 ml of the cell suspension was taken from each vessel into each test tube in which 5.0 ml of the hypotonic solution was mixed; the four test tubes were arranged in a line extending north and south. The cell suspension was taken from I vessel into I test tube which was placed at the northern extremity and from IV vessel into test tube at the southern extremity.

The four curves, I, II, III and IV, in Fig. 3 were obtained with the cell suspension contained respectively in vessels, I, II, III, and IV. These curves are inclined to show similar time-fluctuations, but the differences among them cannot be disregarded, showing that the features of the fluctuation are not identical with the positions, I, II, III, and IV, where the vessels were located. Curves are complicatedly intersected, that is an evidence that the X-agent was changing in different ways according to the different places presumably not only in quantity but also in quality.

Experiments carried out using a red cell suspension contained in different vessels placed under extremely different conditions

As described above, the time-fluctuation patterns were found to be considerably different even with blood cells contained in separate vessels arranged around the same water bottle. Hence, it is only natural that the difference should become much more pronounced when the vessels were placed under conditions made purposely dissimilar as shown in Fig. 4.

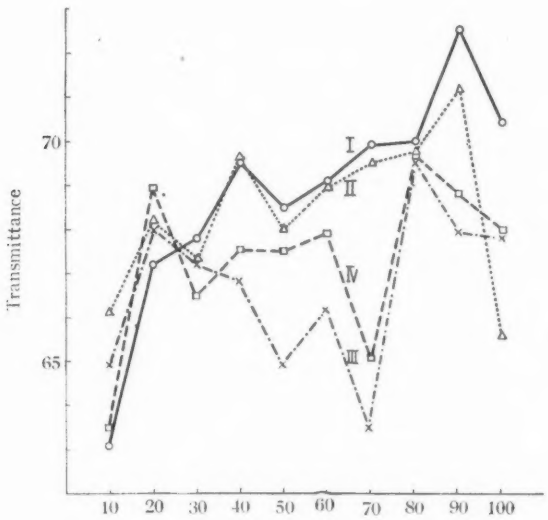


Fig. 4. Fluctuation of liberated hemoglobin amount.
Date of experiment : April 1, 1959.
Curves I and II were obtained with red cells contained in uncovered glass vessels. Curves III and IV were obtained with red cells contained respectively in a glass vessel covered with lead plates and in a vessel placed in a brass box.

Curves I and II in this figure were attained with the cell suspension contained in two glass vessels placed in the same manner as in the experiment shown in Fig. 2, but the vessel with which curve III was obtained was covered with 3 sheets of lead-rubber plates whose thickness was about 0.2 cm, and the vessel with which curve IV was acquired was placed into a brass box with a wall of about 0.2 cm in thickness. These vessels were placed 50 cm apart on a table after they were thus treated.

At intervals of 10 minutes, 1.0 ml of the cell suspension was removed from each of the vessels and put into every one of the four test tubes which had previously been provided with 5.0 ml of the hypotonic salt solution; the tubes were arranged in a row on the same stand and after the completion of the hemolysis they were subjected to centrifugation.

Curves I and II are resembling because they were obtained in the same manner as in Fig. 2, whereas curves III and IV are very different from the former two curves, presumably because they were obtained with blood cells contained in vessels covered with metals. It may be note-worthy that curves III and IV appear to be somewhat similar, suggesting that both metals may be similar in the influence upon the X-agent.

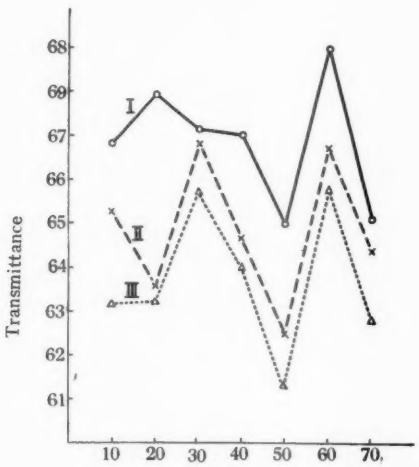


Fig. 5. Effect of NaCl immediately after its dissolution.
Date of experiment : March 24, 1959.
Curve I was obtained with a hypotonic solution of NaCl which was dissolved just prior to the investigation.
Curves II and III were obtained with a hypotonic solution made by mixing 0.9% NaCl solution with water.

Peculiar effect of hypotonic salt solutions immediately after the dissolution of NaCl

All the experimental results presented so far were obtained using hypotonic salt solutions prepared by mixing 0.9 per cent NaCl solution, which had been kept in store, with a certain amount of water. However, when a hypotonic solution was used which had been prepared by dissolving NaCl just prior to the experiment, the result would reveal a different feature.

Thus, curve I in Fig. 5 was obtained using 0.35 per cent NaCl solution which had been prepared by dissolving the salt just before the experiment, while curves II and III were acquired using a hypotonic solution of the same concentration prepared by mixing 0.9 per cent NaCl solution with water. 1.0 ml of the red cell suspension contained in the same vessel was taken into every 3 test tubes, the first of which contained 5.0 ml of a hypotonic solution prepared directly by dissolving the salt, the second and the third containing the same volume of a solution made by mixing 0.9 per cent salt solution with water. The three tubes were arranged side by side on the same stand. The removal of the cell suspension was done at intervals of 10 minutes, being initiated 10 minutes after salt dis-

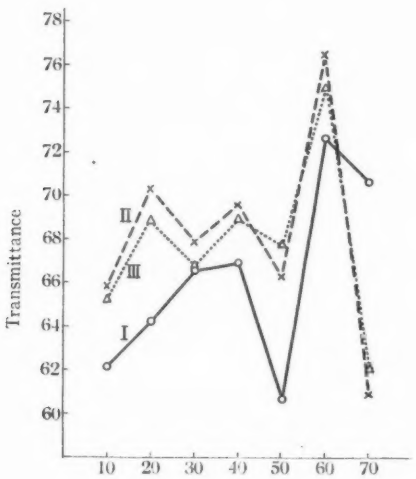


Fig. 6. Effect of NaCl immediately after its dissolution.
Date of experiment : March 23, 1959.
Curve I was obtained with a hypotonic solution of NaCl which was dissolved just prior to the investigation.
Curves II and III were obtained with a hypotonic solution made by mixing 0.9% NaCl solution with water.

solution.

It is evident that curve I in Fig. 5 is different from the other two curves, although they become similar 30 or 40 minutes after the dissolution of the salt. Another example is shown in Fig. 6; an analogous phenomenon is seen likewise in this figure.

These results indicate that the hypotonic solution, if prepared directly by dissolving NaCl, showed a property different from that prepared by mixing 0.9 per cent NaCl solution with water, but that the peculiar property was lost 30 or 40 minutes after the dissolution of the salt.

In order to know whether or not the X-agent was involved in this phenomenon, experiment shown in Fig. 7 was made.

It was considered not to be impossible that the X-agent previously absorbed by NaCl, if such occurred, might be liberated when the salt was dissolving, resulting in the disturbance of the fluctuation curve. If such was the case, proper manipulations applied to NaCl might change the result. Following such a postulation, to a usual NaCl preparation (Teikoku-seiyaku Co.) two sorts of manipulations were added, one of which consisted in its heating on a Bunsen-lamp for 30

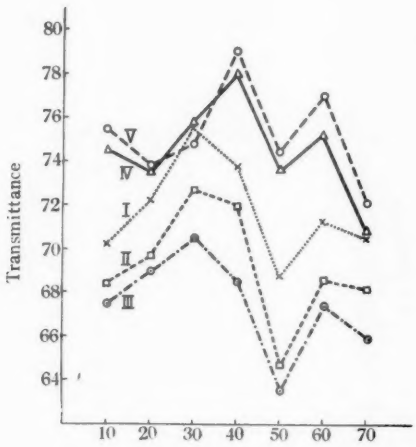


Fig. 7. Effect of NaCl immediately after its dissolution.
Date of experiment : March 25, 1959.
Curves I, II, and III were obtained with hypotonic solutions made with various NaCl preparations which were dissolved just prior to the investigation.
Curves IV and V were obtained with a hypotonic solution made by mixing 0.9% NaCl solution with water.

minutes and the other consisted in its exposure to the direct sun beam for the same time interval, and the salts thus treated were dissolved just prior to the experiment. Curve I was acquired with the former salt and curve II with the latter, while curve III with untreated salt. Curves IV and V were attained with a solution made by mixing the saline with water.

It is evidently shown that the three curves, I, II, and III, are similar, but they are different from the two curves, IV and V. This indicates that the peculiar property of NaCl was not changed by the treatments. It will again be noted that the two groups of the curves are thus dissimilar at the beginning, but later they come to resemble. The difference in the position of the curves is attributable to the difference in the position of test tubes in which hemolysis took place.

If the X-agent was being emitted from NaCl immediately after the dissolution, a test tube of red cell suspension placed in a glass vessel containing a solution of NaCl which had been just dissolved would present a different feature from that placed in a solution of NaCl which had been dissolved for a long time. However, no essential difference was found between the two suspensions even when NaCl was dissolved in so high a concentration as 20 per cent.

Thus, the postulation that the X-agent might be involved in this phenomenon failed to be ascertained. The author is inclined to think that this is caused by a change occurring in the molecular structure of NaCl after the dissolution. Such a concept may seem to be unreasonable, since the structure of NaCl is commonly thought to be extremely simple. However, evidence has accumulated to show that salts, such as Na phosphate, AgCl, or various metal sulfides, are changing in their structure after they have been produced in a solution[5]; the change has been found in general to be finished in an hour or so.

If the structure of NaCl was changing after the dissolution, its effect upon red cells would be changed with it, disturbing the time-fluctuation curve of the X-agent, and as the change would be finished in about an hour, a hypotonic solution which was prepared by dissolving NaCl before an hour or so, would give a result similar to that obtained with a saline solution prepared by mixing the saline with water.

DISCUSSION

Evidence has been presented that the X-agent is changing with time incessantly. The evidence could be attained only when blood cells were adopted as the experimental object.

Since a considerable long period of time is required for the bacterial growth, it is difficult to confirm this fact with the bacteria. On the other hand, the change in the turbidity of a protein solution may be a reflection of the time-fluctuation of the X-agent. However, the change is not fixed and the turbidity may be changed during the short time of estimation. Ordinary proteins may be thus

not appropriate to demonstrate the presence of the time-fluctuation of the agent. Nevertheless, as a protein is subject to the influence of the agent which is changing constantly, proteins and also all the living organisms composed of proteins must be subject to a constant change.

For many years the author has attracted attention to the fact that the process of inactivation of some physiologically active proteins, such as enzymes and viruses, by appropriate inactivating substances would proceed in an extremely irregular manner[6][7]. The inactivation proceeded occasionally with a remarkable oscillation as if it were a physical phenomenon. It was demonstrated likewise that the turbidity produced in an ovalbumin solution by the addition of tannin showed a similar oscillation in the progress of time following the addition[8].

The author once believed that such an oscillation phenomenon could be accounted for by the reversibility of protein structures, as follows: Since protein molecules possess the structural reversibility, viruses or enzymes inactivated to a certain extent may be striving to resume their original, active structures in resisting the inactivating action of the added inactivating substances; these two opposite actions may produce the oscillation.

However, now it has become clear that this phenomenon, at least in most cases, is presumably caused by the X-agent undergoing a constant oscillation.

Furthermore, the author has found that protein particles, including viruses, when suspended in a sugar or a salt solution, could sometimes absorb and sometimes excrete the solute[9][10]. This was thought to be dependent upon the adsorption and elusion of the solute according to the oscillating change in the protein structure. This absorption and excretion phenomena should be expected, since the structure of the protein particles is changing synchronizing with the X-agent, and the change may cause sometimes adsorption and sometimes elusion.

The X-agent is not only changing with time, but also with space. Even when a series of test tubes containing blood cells, or a protein solution, or a culture solution of bacteria, are arranged side by side in a line on the same stand, each test tube will develop each different feature, and the pattern of the different features of test tubes varies with the direction in which the test tubes are arranged[4].

In our routine methods of various investigations, frequently a group of test tubes may be arranged side by side on the same stand. For instance, in an investigation of the formalin effect upon a phage, a number of test tubes containing a series of various concentrations of formalin may be arranged side by side and a given quantity of the phage may be added to each of them. But, since the effect of the X-agent varies with each test tube no regular relationship between the formalin concentration and the degree of phage inactivation will be obtained. In reality, the author has found it sometimes to be very irregular[11].

The potency of immune sera is as a rule examined in this manner, and so

we will encounter very frequently with extremely annoying results. Moreover, there seems to be a storm in the X-agent from time to time[1]. In such a storm the results will be most perplexing. For instance, as often observed, Widal reaction would be found positive in a test tube in which only a trace of the immune serum was contained, while in another tube in which a greater amount of the serum was present the reaction would seem negative.

Such an irregularity is the essential feature of the X-agent, and since proteins and all the organisms are subject to the effect of this agent, we can never avoid the irregularity in the study of living things. This irregularity may seem unpleasant and even absurd but the nature of life should consist in this irregularity, and this nature must be based upon the irregularity of the X-agent governing the living things.

However, it is very strange that most researchers studying proteins and their related substances, including living things, have failed to observe this irregularity. Almost all the data presented so far have been tidy and beautiful. This must indeed be a miracle.

As emphasized above, the essential feature of the X-agent consists in its ever-changing character, changing unceasingly with time and space. For this reason, a datum concerning the agent obtained at a certain time cannot, in general, be obtained twice in the same figure, even when the investigation is made in the same place. In other words, an experimental result once acquired fails to be reproduced in a strict sense. On the other hand, reproducibility is believed to be a fundamental and indispensable criterion in the field of natural science, so that any datum lacking reproducibility is disregarded as incredible. Even a worker, who obtained a datum lacking reproducibility which, however, might naturally be expected if the existence of the X-agent was accepted, might disregard his own experimental result as only a product of a technical failure. On the contrary, if a worker was so self-confident and courageous as to publish his data, as was the present author, not only the data would be ignored but also the worker himself would be regarded as an unskilled, unreliable researcher.

Most probably this may be the reason why the presence of the X-agent has never been acknowledged upto the present, despite the fact that the agent is operating in everywhere and at any time, exhibiting various features in almost all the phenomena concerning living things.

The X-agent is thus playing most amazing part in the living world of the present day. The stage of its operation, however, is presumably not confined to the present time, but it may have been playing active part since the primeval periods of the earth. According to the author's concept[6], the life was generated and evolved in protoplasm-like masses consisting of proteins and lipids, a model of which the author has been able to produce *in vitro*[6][12][13][14]. The X-agent must have had its influence also upon such protoplasm-like masses in the primeval ages.

Since the influence would fluctuate rather rhythmically with time, the masses would have to be undergoing a fluctuating change synchronizing with the X-agent, resulting in the revelation of the primitive feature of absorption and excretion as stated above. Even primitive motions, although they must have been passive, were most probably developed in this manner.

At present, we can follow by a slow-motion picture complicated movement of some plant portions such as leaves and petals. Most of such motions, at least, must be caused by the X-agent fluctuating in a complicated manner. In fact, Stoppel[15] believed that the rhythmical motion of plant seedlings are caused by an unknown external factor. She observed that plants, imported from different places and germinated under constant conditions, gave rise to seedlings which had rhythms in phase with one another. The motion must have been synchronizing with the fluctuation of the X-agent.

Thus, without the effect of fluctuating character of the X-agent, the development of life itself might be extremely difficult, if not impossible. Even the human evolution in the present day appears to be seriously subjected to a climatic factor.[6] Most probably the X-agent is involved in this, as discussed previously[3].

SUMMARY

It has been confirmed using red blood cells that the X-agent is changing unceasingly with time, presenting fluctuations in both quantity and quality.

Evidence was presented that various proteins, such as enzymes and viruses, are changing constantly with the X-agent.

The X-agent is changing not only with time but also with place, so that proteins, including living things, are to be changed with both time and place, resulting in the revelation of an unsystematic irregularity in the experimental data.

References

1) Moriyama, H., Tohoku J. Exp. Med., 1961, **73**, 127.
2) Moriyama, H., Japan. J. Microb., 1960, **4**, 83.
3) Moriyama, H., Japan. J. Microb., 1960, **4**, 7.
4) Moriyama, H., Japan. J. Microb., 1960, **4**, 229.
5) Moriyama, H., Immunity. Igaku-shoin Co., Tokyo, Japan, 1955.
6) Moriyama, H., The Nature of Viruses and the Origin of Life. Igaku-shoin Co., Tokyo, Japan. 1955.
7) Moriyama, H. & Ohashi, S., Zeit. Immun-forschung, 1941, **99**, 349,
8) Moriyama, H., J. Shanghai Sci. Inst., 1937, **3**, 109.
9) Moriyama, H., Archiv Virusforschung, 1940, **1**, 430.
10) Moriyama, H., Archiv Virusforschung, 1940, **1**, 517.
11) Moriyama, H. & Ohashi, S., Archiv Virusforschung, 1939, **1**, 252.
12) Moriyama, H., Yokohama Medical Bulletin, 1958, **9**, 323.
13) Moriyama, H., Yokohama Medical Bulletin, 1959, **10**, 39.
14) Moriyama, H., Jap. Jour. Exp. Med., 1959, **29**, 9.
15) Stoppel, R., Zeit. Bot., 1916, **2**, 609.

Tohoku J. Exper. Med., 1961, 73, 159–169

Electromyographic Studies on Urinary Tract

III. Influence of Pinching and Cutting the Ureters of Dogs on Their EMGs

By

Tsuneo Shiratori and **Hideo Kinoshita**

From Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, August 30, 1960)

INTRODUCTION

In sequence to the previous study, the ureters of dogs were pinched or cut under laparotomy, and their EMGs were recorded under such conditions. The results obtained are remarked in the present report.

EXPERIMENTAL METHODS

Thirty two adult dogs were subjected to the experiments described below under the same conditions as given in our second report[1]. Their ureters were pinched or cut at the pyeloureteral and ureterovesical junctions and at the middle part of the ureter.

EXPERIMENTAL RESULTS

I. EMG of pinched ureters

1) When the ureters were pinched at the pyeloureteral junction in the 5 cases, as shown in Fig. 1, left side and Fig. 2, one normoperistaltic discharge was set off at the pinching, then the peristaltic discharge was suspended for 1.0–2½ min. before the spontaneous normoperistaltic discharges were reinstated.

The pinching little affected the frequency, the amplitude, the duration or the wave pattern.

2) When the ureters were pinched at about the median point in 5 cases, as shown in Fig. 1, middle portion and Fig. 3, upward and downward propagating discharges occurred simultaneously at each pinched part. Thereafter, normoperistaltic discharges continued to occur in the part above the clamp, but in the part distal to it, peristalitc discharges were in abeyance for 1½–2½ min. before the normal discharges came through the pinched part. Here again, the properties about the discharges were not perceptibly affected by the pinching.

白鳥常男，木下秀雄

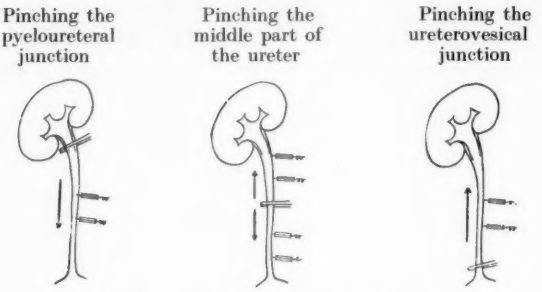


Fig. 1. The ureters were pinched at three points.

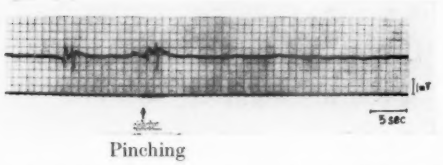


Fig. 2. EMG by pinch at the pyeloureteral junction.

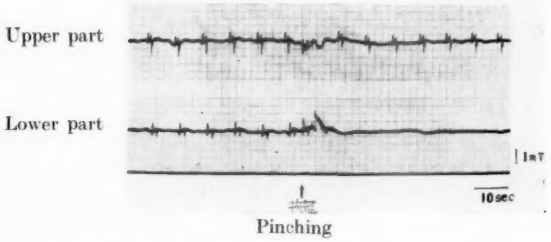


Fig. 3. EMG by pinch at the middle part of the ureter.

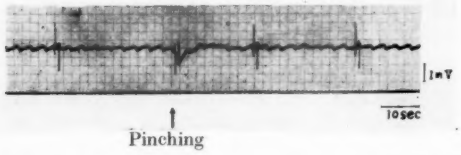


Fig. 4. EMG by pinch at the ureterovesical junction.

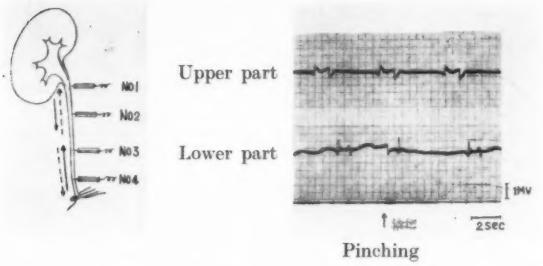


Fig. 5. EMG led off with two pairs of bipolar leading by pinch at the ureterovesical junction when the spontaneous normoperistaltic discharges reappeared.

3) When the ureters were pinched at the ureterovesical junction in 7 cases, as shown on the right of Fig. 1 and Fig. 4, only a single antiperistaltic discharge arose at the pinched part, the normal normoperistaltic discharges from above being neither suspended nor perceptibly changed in their propagation velocity and such properties.

In the experiment with two pairs of bipolar leading, as shown in Fig. 5, when the ureter was pinched at the ureterovesical junction at the time of appearance of normoperistaltic discharges at the upper twin electrodes, the antiperistaltic discharges set off from the pinched part were detected only by the lower pair of electrodes without being transmitted to the upper pair, while the normoperistaltic discharge from above faded out before reaching the lower pair, so that it is clear that the two opposite discharges collided and cancelled out each other midways.

II. EMG of cut ureters

1) When the ureter was cut at the pyeloureteral junction in 6 cases, as shown in Figs. 6 and 7, the normoperistaltic discharges of 3–14 w/min. observed before the cut were stopped at once, and 16 sec.–4 min. 25 sec. thereafter, antiperistaltic discharges of 0–7 w/min. began to be set off from the ureterovesical junction and remained active throughout the period of observation for 17–50 min. In 2 of the 6 cases, these antiperistaltic discharges came forth as solitary discharges at irregular intervals ranging between 13 sec. and 187 sec. (Fig. 7), in one case, they appeared at the group-intervals of 32–111 sec. in groups of two or three discharges of 1.8–2.3 sec. intervals, and in the other three cases at the irregular intervals of 18–232 sec. as solitary discharges mingled with groups of 2–7 discharges (Fig. 8).

In these cases, the measured values of the propagation velocity, the amplitude, the duration and the wave pattern were 20–45 mm/sec., 0.3–3.4 mV., 0.2–0.9 sec. Type I or II of normoperistaltic discharges and 27.3–45 mm/sec. 0.3–

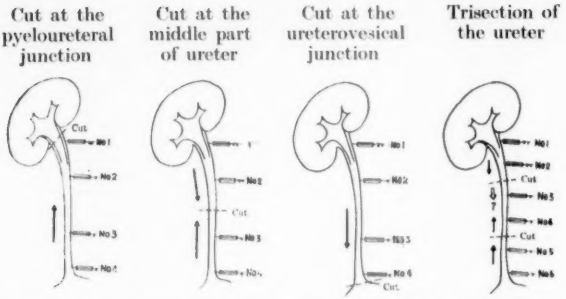


Fig. 6. The ureters were cut at parts indicated by broken line.

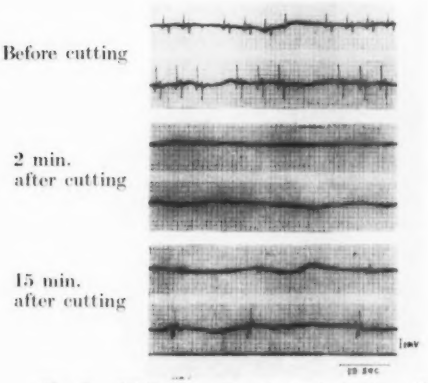


Fig. 7. EMG led off with 4 electrodes after cut at the pyeloureteral junction.

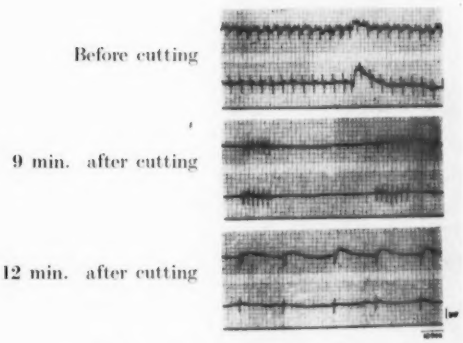


Fig. 8. EMG led off with 4 electrodes after cut at the pyeloureteral junction.

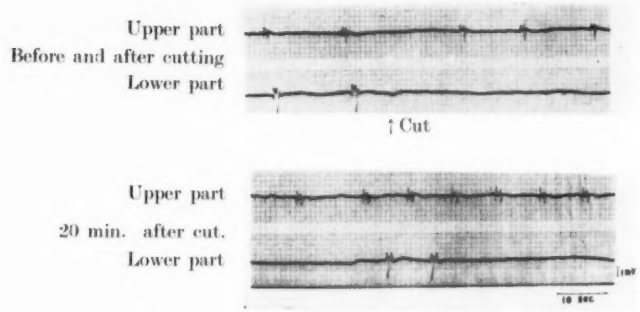


Fig. 9. EMG after cutting the middle part of ureter.

3.4 mV., 0.2–0.8 sec. I or II of antiperistaltic discharges, respectively.

2) When the ureter was cut off midways in 8 cases, the normoperistaltic discharges of 4–13 w/min. in frequency coming from above to the cut point were nearly the same frequency as those before the cutting (2–17 w/min.) as shown in Fig. 9.

In the ureter below the cut, the normoperistalitc discharge was suspended after the cutting, transiently in 4 cases, in which antiperistaltic dischages of 0–10 w/min. in frequency began to be set off in 50 sec.–3 min. from the ureterovesical junction and remained in force during the period of observation for 10–28 min., but in the remaining 4 cases, we could not observed such upward discharges.

In 2 of the 4 cases with the antiperistaltic discharges, they were grouped in 2–10 discharges at 1.8–3.2 sec. in intervals, the groups occurring at the intervals of 84–176 sec., in other cases, they occurred in solitary discharges at the irregular intervals fluctuating between 10 sec. and 79 sec. and in another case, solitary and grouped discharges appeared in mixture at the intervals of 44–152 sec.

The propagation velocity, the amplitude, the duration and the wave pattern of these normoperistaltic and antiperistaltic dischagres above and below the cut were summarized in the following Table. I.

In the cases in which no discharge was found to occur in the ureter below the cut, i.v. administration of 10 mg/kg. of 2 % xylocaine caused occurrence of antiperistaltic discharges of 0–8 w/min., as shown in Fig. 10, and the frequency of the normoperistaltic discharges in the part above the cut was raised thereby from 5–8 w/min. to 20–30 w/min. A similar injection in the case with antiperistaltic discharges of 1–3 w/min. caused an increase of the frequency of both the normoperistaltic discharges above the cut and the antiperistaltic discharges below it, as shown in Fig. 11.

TABLE I. The Measured Values of Discharges before and after the Cutting

	Direction	Propagation velocity mm/sec.	Amplitude mV.	Duration sec.	Wave pattern
Upper ureter	Before cut. Normo.	22.7–46.9	0.3–1.45	0.25–0.6	I–II
	After cut. Normo.	22.7–44.1	0.3–1.5	0.3 –0.55	I–II
Lower ureter	Before cut. Normo.	16.6–46.2	0.3–2.5	0.25–0.65	I–II
	After cut. Anti.	23.2–46.2	0.4–2.6	0.3 –0.55	I–II

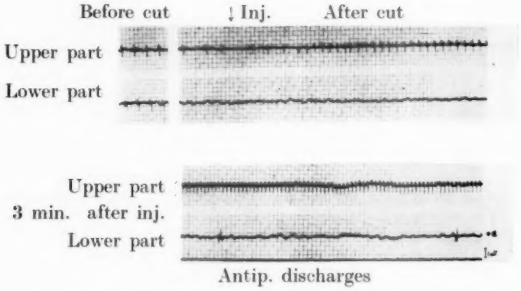


Fig. 10. EMG by i.v. injection of 2% xylocaine 10 mg/kg. in a case in which discharge disappeared following cutting at the middle.

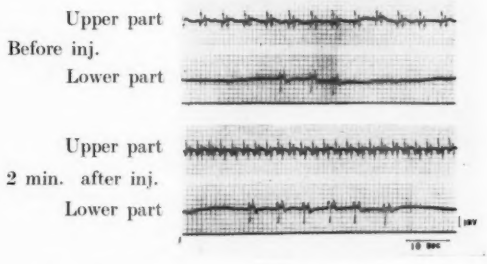


Fig. 11. EMG by i.v. injection of 2% xylocaine 10 mg/kg following cutting at the middle part of the ureter.

3) When the ureter was cut at the ureterovesical junction in 5 cases, two pairs of bipolar leading placed in the upper part of the ureter continued to record the discharges (2–8 w/min.) of the same frequency as before the cutting (2–9 w/min.) throughout the period of observation for 26–52 min. (Figs. 6 and 12). Their propagation velocity, amplitude, duration and wave pattern were not

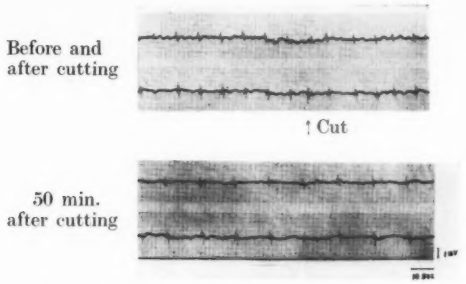


Fig. 12. EMG by cut at the ureterovesical junction.

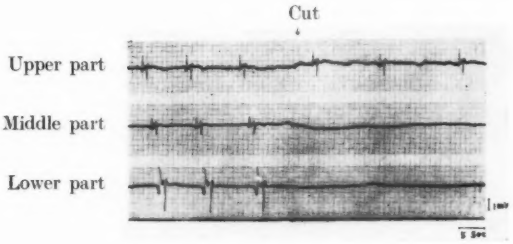


Fig. 13. EMG by trisection of ureter.

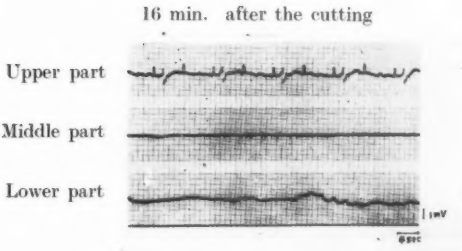


Fig. 14. EMG of trisected ureter. The direction of the discharge found at the middle part was unknown.

perceptibly affected by the cutting either.

4) When the ureter was trisected lengthwise in 5 cases, the normal normoperistaltic discharges persisted at unvarying frequency (3–9 w/min. before and 4–9 w/min. after the trisection) in the upper 1/3, as shown in Fig. 13. In the median 1/3, we found no discharge occurring at all in 3 cases throughout the period of observation for 20–45 min., while in the other 2 cases, as shown in Fig.

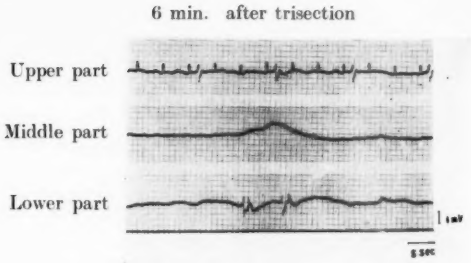


Fig. 15. EMG of trisected ureter. In the lower part antiperistaltic discharges were found.

14, discharges suggestive of antiperistalitc direction were caught by the lower electrode in 28 and 107 sec.; in one of them only two groups of three discharges each at 3–3.5 sec. of intervals appeared at the group-interval of 13 min. apart (0–3 w/min.), and in the other 14 solitary discharges came forth at the irregular intervals of 28–145 sec. (0–2 w/min.).

In the lowest 1/3 of the cut ureter, 6–17 antiperistaltic discharges appeared in 4 cases 128–272 sec. after the trisection, at the irregular intervals of 6–252 sec. and the frequency of 0–4 w/min. continuing throughout the period of observation of 20–45 min. as shown in Fig. 15. In the other case we observed no occurrence of such discharges.

The particulars of the discharges before and after the triscetion were as follows (Table II).

In the cases where discharges failed to occur in the middle or the lowest part, i.v. injection of 10 mg/kg. of 2 % xylocaine caused antiperistaltic discharges of 1–4 w/min. in all the cases except in one middle portion, as shown in Fig. 16. In the upper 1/3, where the normoperistaltic discharges always persisted after the trisection, the frequency of the discharges was more than

Table II. The Measured Values of the Discharges by Trisection

	Prppagation velocity mm/sec.	Amplitude mV.	Duration sec.	Wave pattern
In the upper 1/3				
Before cut	13.3–26.8	0.4 –3.0	0.3 –11	I–II
After cut	13.3–27.6	0.4 –2.6	0.35–1.1	Ditto
In the middle 1/3				
Before cut	27.0–34.1	0.2 –1.5	0.5 –0.62	Ditto
After cut	Ditto	0.35–1.1	0.15–0.2	Ditto
In the lower 1/3				
Before cut	21.4–29.7	0.4 –3.3	0.4 –0.9	Ditto
After cut	29.7–40.6	0.4 –2.9	0.6 –0.9	Ditto

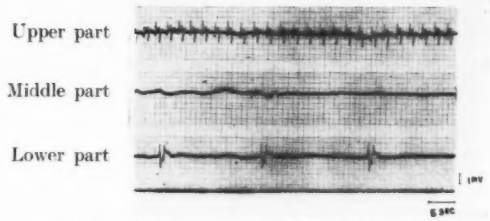


Fig. 16. EMG by i.v. injection of 2% xylocaine 10 mg/kg. in those cases in which the discharge at middle and lower parts faded out following the trisection.

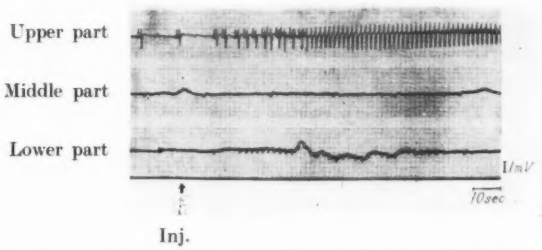


Fig. 17. EMG by i.v. injection of 2% xylocaine 10 mg/kg. in those cases in which the discharge appeared at any parts following the trisection.

quadrupled by similar injection. A marked increase in frequency was observed also after similar injection in the case with discharges appearing in the middle and the lower part after trisection, enduring for a few min., as shown in Fig. 17. The increase in frequency and continuing time of the discharges was the largest in the upper 1/3, next in the lower and the smallest in the middle portion.

DISCUSSION

Alksne[2], Gruber[3], Wu[4], Nobuta[5], Takagi[6] and Satani[7] have reported on experiments with cut ureters, but these studies were conducted with excised ureters. We have made systemic experimental studies following pinching and cutting of vital ureters of dogs at different points and described the EMG records of the upper, the middle and the lower parts of the ureters in the above.

We found that a single normoperistaltic discharge occurred upon pinching the pyeloureteral junction, a pair of normo- and anti-peristaltic discharges upon pinching the middle part and a single antiperistaltic discharge upon pinching the ureterovesical junction, always at the pinching pcint. These findings are in complete concurrence with those by Engelmann[8] and Alksne[2]. After pinching of

the pyeloureteral junction and the middle part of the ureter, the transmission of discharges was suspended for a few mimutes.

In the experiments with cut ureters, we found that the ureter, even if bisected or trisected, has a capacity for contraction in any of the cut portions, and the pyeloureteral junction and the ureterovesical junction have each a direction-determining factor, the normoperistaltic wise and the antiperistaltic wise respectively, but the middle portion failed to show any definite direction determination.

Gruber[3] experimented with excised ureters and found the mean frequency of contractions to be the highest in the upper portion (4.1 w/min.), next in the lowest portion (3.4 w/min.) and the smallest in the middle portion (2.0 w/min.), Alksne[2] made similar experiments with excised ureters of gunea pigs and reported the frequency of contractions per 5 min. of 4–6 in the upper portion, of 3–6 in the lowest portion and 1–4 in the middle portion. Our findings with trisected ureters seem to accord with these authors'. But Wu[4], Nobuta[5], Takagi[6] and Satani[7] who experimented with excised animal ureters arrived at results at variance with ours, claiming that the frequency of peristaltic movements was the highest in the upper, next in the middle and the lowest in the lowest portions.

Hryntschak[9] found upon resecting at the pyeloureteral junction of pig's ureter excised with a part of the urinary bladder attached to it, the original normoperistaltic movement was reversed into the antiperistaltic direction. Alksne[2] cut vital canine ureters and found antiperistaltic movement always arising at the vesical end in the lower portion of the ureter. Takagi[6] reports that in the ureters cut out from dogs, guinea pigs and rabbits without detaching the pyeloureteral junction, the peristalsis always runs downwards, and only very rarely upwards from the vesical end, but in the samples of ureter excised from the pyeloureteral junction, the antiperistaltic movement was usual. These findings are apparently in general concurrence with what we found in our cut ureters.

Gruber[3] investigated the spontaneous movement of bisected ureters sampled from pigs and said that the relative frequencies of observed normo- and antiperistaltic movements were 73.7% : 26.3% in the renal half and 28.1% : 71.9% in the vesical half, but these figures are apparently irreconciable with our results, for we saw only normoperistaltic movements in the renal half and only antiperistaltic movements in the vesical half of cut ureters.

The results of the experiments with cut ureters above and our previous paper showing that the frequency and the increment of frequency of the discharges following xylocaine injection in the periureteral tissues are both the highest in the renal part of the ureter indicate that the pyeloureteral junction is the most sensitive to stimulation of all the ureter and it is probable that this part plays a leading part in the maintenance of physiological normoperistalsis.

CONCLUSION

The ureters of 32 adult dogs were exposed by laparotomy, pinched or cut at pyeloureteral junction or ureterovesical junction or some intermediate points and their EMG was studied. The results obtained were in summary as follows:

1) When the ureter was pinched at the pyeloureteral junction or the ureterovesical junction, one single normo- or anti-peristatlic discharge each was set off from the respective pinched part, while the clamp was applied to the middle part of the ureter, one upward antiperistaltic and one downward normoperistaltic discharges were simultaneously released from the pinched part. When the pyeloureteral junction or a mid-ureteral point was pinched, a suspension for $1\frac{1}{2}$–$2\frac{1}{2}$ min. of discharge transmission occurred in the parts below the clamp.

2) Even after the ureter was bisected or trisected, the individual portions all showed spontaneous contraction and emitted peristaltic discharges, in the normoperistaltic direction in the upper portion, the antiperistaltic direction in the lower and in the antiperistaltic or suspiciously antiperistaltic direction in the middle portions. The frequency of these partial spontaneous discharges was the largest in the upper portion (2–13 w/min.), next in the lower portion (0–10 w/min.) and the smallest in the middle portion (0–3 w/min.).

3) Following i.v. administration of 10 mg./kg. of 2% xylocaine in the cases showing occurrence of discharges in all the portions of bisected or trisected ureters, the frequency of discharges was always raised in any portion, and in the case where no discharge came forth in the middle and the lower portions, such an injection caused the appearance of antiperistaltic discharges in the lower portion and suspiciously or clearly antiperistaltic discharges in the middle portion. The frequency and the continuing time of discharges after the injection were the largest in the upper, next in the lower and the smallest in the middle portions.

4) It was concluded that the pyeloureteral junction plays a leading part in the maintenance of physiological peristaltic function of the ureter and that the lower portion of the ureter severed from this junction shows a strong tendency of releasing antiperistaltic movements.

References

1) Shiratori, T. & Kinoshita, H., Tohoku J. Exp. Med., 1960, **73**, 103.
2) Alksne, J., Folia Urol., 1907, **1**, 338.
3) Gruber, C.M., J. Urol., 1928, **20**, 27.
4) Wu, P.P.T., J. Urol., 1933, **66**, 307.
5) Nobuta, S., Jap. J. of Urol., 1925, **14**, 119.
6) Takagi, M., Acta. Med., **7** (Jap.), 831, 1933.
7) Satani, Y., Amer. J. Physiol., 1919, **49**, 474.
8) Engelmann, J.W., Arch. f. d. ges. Physiol., 1869, **2**, 243.
9) Hryntschak, T., Arch. f. d. ges. Physiol., 1925, **209**, 542.

Tohoku J. Exper. Med., 1961, 73, 170–179

Electrical Properties of Glycerinated Crayfish Muscle Fiber

By

Shiko Chichibu

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, August 31, 1960)

INTRODUCTION

The study of the mechanism of the muscle contraction has been made on the glycerol-extracted muscle fibers mainly with mechanical recording methods.[7][8] And it is the well known fact that vertebrate muscle contains contractile protein compound–actomyosin, and that the shortening of extracted muscle is the result of actomyosin-ATP reactions. On the other hand, the living muscle cell shows an action potential when they excited, and the site of action potential generation is considered to be the protoplasm membrane of the muscle cell. The combination of these two phenomena is said to be the excitation-contraction coupling of muscle.

There have been a great number of investigations on the mechanical properties of the glycerol-extracted muscle, but very few on its electrical properties. In 1950 Korey reported that a perfectly glycerol-treated rabbit muscle did not contract in response to direct stimulation of muscle.[6]

The object of this paper is to deal with the electrical properties of glycerol-treated crayfish muscle which are exhibited during a transient period from the initial to the final state of treatment.

METHOD

Extensor muscle of a walking leg of the American freshwater crayfish (Procambarus clarkii GERARD) was used. The extensor of carpopodite was exposed in crayfish saline in the same way as described by Fatt & Ginsborg (1958).[1] A preparation was mounted in a metacrylate resin chamber filled with physiological saline. Both ends of the preparation were clamped with small forceps and it was fixed in a slightly extended state. A period of rest of about 15 minutes was allowed before the experiment to avoid the quasi-stationary state caused by the operation.

In adult animals the diameter of the muscle fiber ranged from 70 to 180 μ,

秩父志行

with the mean value of 110 μ, as examined with a binocular microscope. Thicker fibers showed a rather opalescent appearance under transmitted illumination, but thinner ones appeared quite transparent. The present experiments were performed chiefly on thinner transparent fibers.

The recording system used was essentially similar to that described by Fatt & Katz (1953)[2], except that sometimes a double-barrelled microelectrode was used, one barrel of which for stimulating and the other for recording at the same point of fiber in measurements of the effective resistance of the membrane. 3M-KCl filled glass micropipettes with tip diameter of less than 0.5 μ and having the ohmic resistance not more than 20 meg-ohms were used for recording as well as for polarization.

The physiological saline solution of crayfish[3] used had the following composition: NaCl 12.0g, $CaCl_2$ 1.5 g, KCl 0.4 g, $MgCl_2$ 0.25 g and H_2O 1000 cc. When a high potassium concentration was needed, sodium chloride was replaced by potassium chloride of equivalent osmotic pressure. The application of glycerol to the preparation was done as follows: Various amounts of pure glycerol were dissolved into normal crayfish saline to obtain glycerol solutions of higher osmotic pressure than normal. Some control experiments were performed with sucrose-van Harreveld solutions of the same osmotic pressures. Replacement of the surrounding solution around the preparation was done by a pair of injection syringes connected to the chamber via rubber tubes.

The experiments were done at the room temperature of 10–15°C.

RESULTS

The glycerol effect on the resting membrane potential

The membrane potential of the crayfish muscle fibers usually ranged between –60 and –87 mV in normal van Harreveld solution with the mean value of –75 mV. When the physiological saline was replaced by glycerol-van Harreveld solutions, potential difference across the membrane decreased with the lapse of time. Fig. 1 A illustrates the time-course of potential decrements. If the muscle was immersed in glycerol solutions for hours, the membrane potential decreased with time and in proportion to the amount of glycerol added to the solution. In cases of high concentration of glycerol the decrease was rapid in the initial stage and more gradual in the later stage. When the concentration was over 500 mM, cell destruction occurred so frequently that it was difficult to obtain reliable results. Concentrated (1–2 M) glycerol solutions caused turbidity in the majority of the muscle fibers. In this state, the cell membrane was very fragile and was easily torn with microelectrode insertion.

Fig. 1 B shows two control observations which were obtained from preparations immersed in sucrose-van Harreveld solutions. In these control ex-

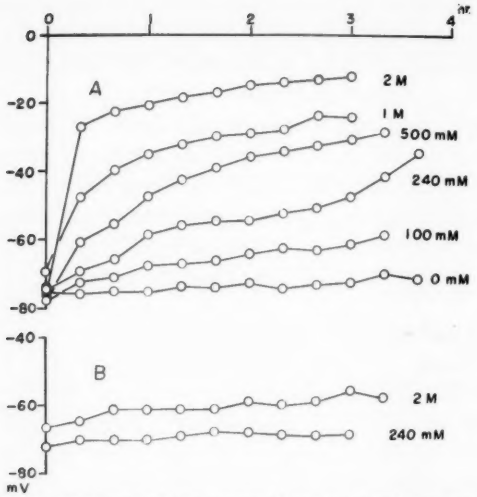


Fig. 1. Decreasing time-course of resting membrane potential in glycerol-van Harreveld solutions (A) and in sucrose-van Harreveld solutions (B). Concentrations of glycerol in A and those of sucrose in B are given on right. Ordinates: Resting membrane potential. Abscissae: Time after application.

periments no remarkable change in resting membrane potential occurred with such a hypertonic solution as the one containing 2 M sucrose.

Diminution of membrane potential also took place when the muscle was immersed into glycerol saline for only a short time. Fig 2 illustrates the decreases caused by a 3 minutes treatment and recovery courses in van Harreveld solution. No recovery was seen with glycerol solutions of higher concentration (500 mM and 2 M), while in case of 100 mM the recovery was almost complete.

The potassium effect on glycerinated muscle cells

In the experiments shown in Fig. 3 the potassium concentration of the external solution was changed. In one experiment the muscle preparation was treated with van Harreveld solution containing 2 M glycerol for about 15 minutes and then the solution was replaced by solutions of various potassium ion concentrations (see the chain line). Similar experiments were carried out with preparations treated with van Harreveld solution containing 240 mM glycerol (see the dotted line). No treatment with glycerol was done in the control experiments (see the continuous line). These experiments indicate that even the 2 M glycerol solution could not destroy the cell membrane perfectly, if the time of application was not long, because the selective permeability for potassium ions

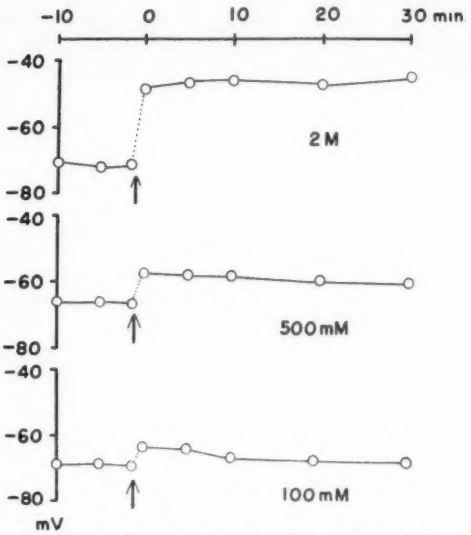


Fig. 2. Effect of glycerol applied for short period. Arrows indicate glycerol application for 3 minutes. Ordinates: Resting membrane potential. Abscissae: Time-course.

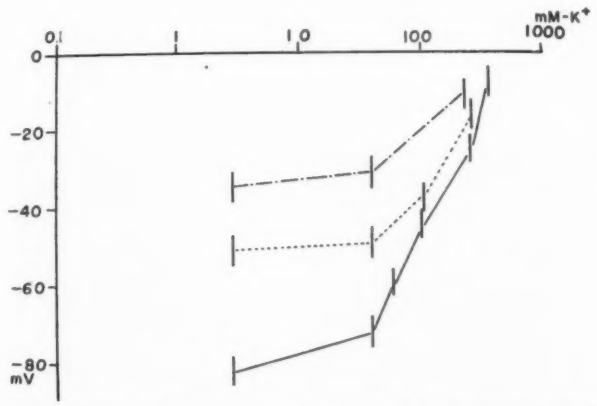


Fig. 3. Relation between external potassium concentration and resting membrane potential. Preparations were conditioned with 2 M glycerol for 15 minutes and 240 mM for 15 min., and control (from upper to lower). Vertical bar indicates range of membrane potential in 3 cases. Ordinates: Membrane potential. Abscissae: External potassium concentration plotted in logarithmic scale.

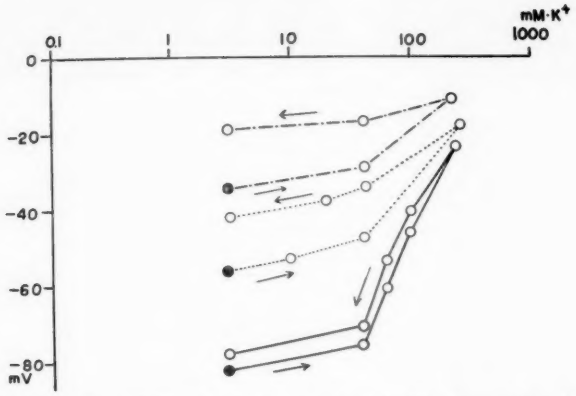


Fig. 4. Reversibility of membrane potential with changes in external potassium concentration. Experiments were performed in direction of arrow. Other explanations as in Fig. 3.

remained in this case.

In Fig. 4, the reversibility was tested for the same kind of experiments as shown in Fig. 3. It can be seen that the reversibility is unsatisfactory in preparations treated with glycerol (see chain and dotted lines) in comparison with the control (see the continuous line).

The glycerol effect on electrotonic potentials

In order to estimate the membrane constants of the glycerinated muscle cell, the rectangular pulse technique was employed. According to the cable

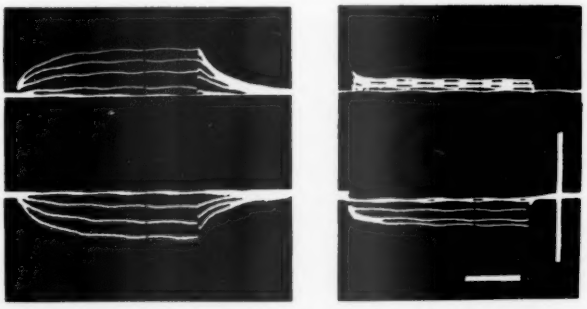


Fig. 5. Electrotonic potentials caused by polarizing currents of various intensities. Before (left) and after (right) application of 500 mM glycerol solution for about 6 minutes. Depolarization upwards, and hyperpolarization downwards. Vertical line: 50 mV. Horizontal line: 10 msec.

theory of Hodgkin & Rushton (1946),[4][5] the effective resistance of the membrane, r_e, can be easily derived from the relation between the current intensity of the applied long-duration pulse and the stationary voltage shift of the membrane potential. The electrotonic potential, V, is given by the following equation,

$$V = \frac{I}{2}\sqrt{r_m r_i}$$

where I, r_m and r_i are the applied current intensity, the transverse resistance of the membrane times unit length, and the longitudinal internal resistance per unit length of the fiber, respectively. The theory demands that the distance between the polarizing and recording electrodes inserted into one and the same locus of the cell is sufficiently small against the characteristic length of the fiber, and that the voltage shift is small and not more than 10 mV.

Electrotonic potentials before and after application of glycerol with weak anelectrotonus and catelectrotonus are illustrated in Fig. 5. As can be seen in this figure, glycerol caused diminution of electrotonic potential changes; the decrease was more marked in depolarization than in hyperpolarization.

TABLE I. Effective Resistances (kilo-ohms) derived from Voltage-Current Relationship before and after Application of 240 mM Glycerol-van Harreveld Solution for 15 Minutes

Fiber	Before application	After application	Room temperature
1	163	103	12°C
2	138	96	13
3	147	103	13
4	155	98	13
5	142	105	14
Mean value	149	101.2	13

Table I shows the results of 5 cases in which 240 mM glycerol-van Harreveld solution was applied for about 15 minutes to crayfish extensors. The effective resistance was decreased to two thirds of the control.

When the intensity of the current was large enough to reverse the resting membrane potential, oscillatory local potentials were usually seen superposing on the electrotonic voltage shift. These local potentials disappeared in a few minutes if the external solution was replaced by glycerol-van Harreveld solution. Fig. 6 illustrates an example.

In Fig. 7, the oscillatory potentials as shown in the control record (the uppermost left record) were gradually reduced in a dilute glycerol solution with time and the potential curves became more simple and smooth. It is to be noted

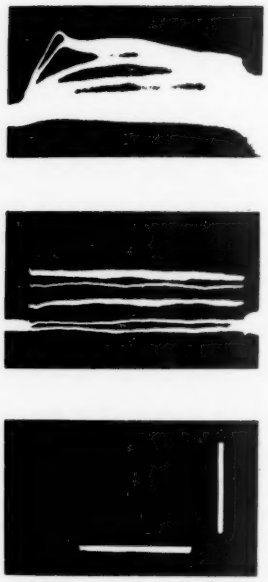


Fig. 6. Glycerol effect on oscillatory action potentials. Before (upper) and after (middle) application of 240 mM glycerol solution for about 15 minutes. Veritcal line : 50 mV. Horizontal line : 20 msec.

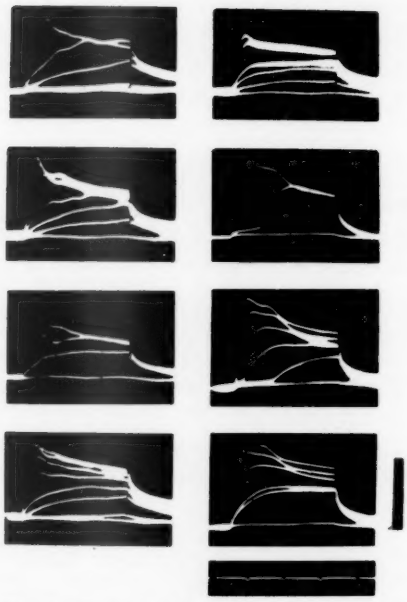


Fig. 7. Changes of oscillatory action potentials superposed on electrotonic potentials with application of 240 mM glycerol. From top to bottom, in left-hand column : control, 1 min., 3 min., and 5 min. after application. In right-hand column, 7 min., 9 min., and 11 min. after application and the bottom record which was obtained 5 min. after replacement of glycerol solution by normal saline. Vertical line : 50mV. Time mark : 10 msec.

that the potential curves obtained with weak depolarizing currents showed a rise in two steps in the transient period (see the second record from right top). It seems as if the normal rise of potential was depressed by some process at the initial stage and then released. When the preparation was washed with normal saline the rise of potential became normal, but oscillatory local potentials were not yet restored (see the right bottom record). Fig. 8 illustrates more distinct examples of delayed rises in potential.

The effect of ATP on action potentials reduced by glycerol

When the innervating axon was stimulated electrically, conducting spike potentials were recorded in muscle cells. This spike potential was also affected by glycerol as shown in Fig. 9. The degree of decrement depended upon the

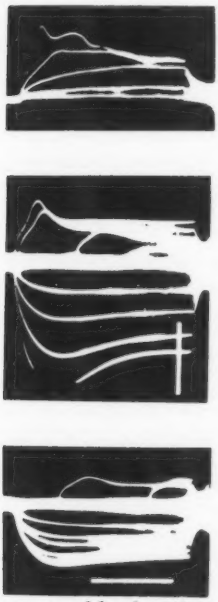


Fig. 8. Delayed appearance of local action potentials with 240 mM glycerol solution. From upper to bottom : control, 2 min., and 5 min. after application. Depolarization upwards and hyperpolarization downwards. Vertical line : 50 mV. Horizontal line : 20 msec.

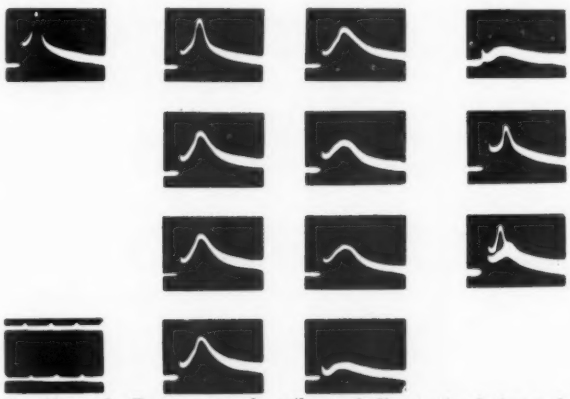


Fig. 9. Glycerol effect on muscle spike to indirect stimulation and recovery by ATP solution. Right-hand column : Control and time mark of 10 msec. Space between two time marks is 50 mV. Middle two columns : 1 min., 2 min., 3 min., 4 min., 5 min., 6 min., 7 min. and 10 min. after application of 240 mM glycerol solution (from right top to left bottom). Left-hand column : 1 min. and 6 min. after application of 0.1% ATP-van Harreveld solution. Spikes obtained before and after recovery are superposed in the bottom record (left-hand column) to make comparison easier.

duration of application. When the concentration was not more than 500 mM, the spike was reduced in amplitude progressively and became as flat as shown in the bottom record of the third column in about 10 minutes. So flattened potentials showed no further change, remaining the same for several hours. No recovery took place even though the external glycerol solution was replaced by normal saline, but sometimes the spike was restored with application of van Harreveld solution containing ATP in concentration of 0.1%, as shown in the right-hand column of Fig. 9.

DISCUSSION

Glycerol affects the resting membrane potential as well as action potential of crayfish muscle. When the application was short and the concentration was not high, the muscle so treated with glycerol exhibited some recovery concerning the resting membrane potential, electrotonic potentials and action potentials to nerve stimulation, though the recovery was not always perfect.

Glycerol decreased the resting membrane potential and the major part of the decrease occurred in the first several minutes of experiment. A dilute glycerol solution caused only gradual diminution in membrane potential, but concentrated one caused a steep depolarization which was followed by a gradual one. The rapid phase would be the result of direct action of glycerol but not the effect of high osmotic pressure on the cell membrane, for the hypertonic sucrose solutions did not show any sign of rapid depolarization.

Glycerol-treated cells showed decreased effective resistances (see Table I). The major part of decrease in effective resistance would be caused by the drop of r_m, the transverse resistance of the membrane.

Crayfish muscle cell rarely produces propagating action potentials by direct stimulation. From this point of view, local responses will be the sign of the muscle fiber excitability. Glycerol depressed the production of local oscillatory potential changes and sometimes caused retardation of local potentials (see Figs. 7 & 8). The diminution of the local potentials was associated with a decrease of the effective resistances of the cell membrane. From these experiments, it may be assumed that the glycerol application would cause the blocking of the excitation process and that the blocking is related to the decrease of membrane resistance.

Generally it is known that ATP has no action on the fresh muscle preparation in contrast to its remarkable effect on perfectly glycerol-extracted muscle. In the last section was demonstrated the recovery of action potentials by ATP application, and similar recovery with ATP is seen in frog skeletal muscle treated with glycerol solutions (unpublished data).

It is possible that ATP is needed for normal functioning of the muscle cell

membrane to produce electrical responses and that glycerol extracts ATP from the cell so that it fails to produce electrical responses. This simplest assumption accounts for the restoration of electrical responses by addition of ATP to the glycerinated muscle.

SUMMARY

Electrical measurements were done on glycerol-treated crayfish muscle cells with intracellular recording.

1. Decrease of the resting membrane potential was caused by van Harreveld solution containing glycerol (100 mM-2 M).

2. Glycerinated muscles responded to the change of external potassium concentration with changes in membrane potential.

3. The effective resistance of the cell membrane was decreased and local action potentials were reduced in glycerinated muscle.

4. The reduced action potential was restored to normal by application of ATP dissolved in van Harreveld solution at concentration of 0.1 per cent.

I wish to express my hearty thanks to Prof. K. Motokawa for his kind advice in this work and in preparing the manuscript. Also I am grateful to Mr. K. Konno of Miyagi Prefectural Agricultural Experiment Station, Iwanuma Branch, for his kindness to supply the crayfishes used in this work.

References

1) Fatt, P. & Ginsborg, B.L., J. Physiol., 1958, **142**, 516.
2) Fatt, P. & Katz, B., J. Physiol., 1953, **120**, 171.
3) van Harreveld, A., Proc. Soc. Exper. Biol. Med., N. Y., 1936, **34**, 428.
4) Hodgkin, A.L. & Rushton, W. A. H., Proc. Roy, Soc., **B**, 1946, **133**, 444.
5) Katz, B., Proc. Roy, Soc., **B**, 1948, **135**, 506.
6) Korey, S., Biochim. et Biophys. Acta, 1950, **4**, 58.
7) Szent-Györgyi, A., Biol. Bull., 1949, **96**, 140.
8) Szent-Györgyi, A., Chemistry of muscular contraction. 1951, Academic Press, N. Y.

Tohoku J. Exper. Med., 1961, 73, 180–190

An Autopsy Case of Primary Chromoblastomycosis Arising from the Internal Organs

Dark-brown Granulomas in the Liver and the Brain without Skin Symptoms, Observed in a Smoking Child 3 Years Old

By

Nobuaki Sasano and **Toyozō Okamoto**

Department of Pathology of Profs. Okamoto and Suwa, Tohoku University School of Medicine, Sendai

and

Takashi Takahashi and **Shōzō Suzuki**

Sennan Hospital, Kakuda-City; Director: Dr. T. Honda

(Received for publication, September 5, 1960)

Chromoblastomycosis is a rare disease in Asia, only 4 cases[7),11),13)] being hitherto reported in Japan. In general, this disease affects the skin, preferentially the skin of the lower extremities, where it is known to cause chronic verrucous or papillomatous granulomas. The cases cited in Carrion's world-wide collection[1)] show only cutaneous symptoms, developing superficially with little or no tendency to involve the deeper structures. Metastases through the blood stream may be produced, but are extremely rare (Carrion & Koppisch[5)]). Nishi[9)] (1956), reported an autopsy case of a girl aged 8 years who had a multiple chromomycosis in her face which metastasized to the brain (which was locally dissected) and had demonstrated presence of *hormodendrum pedrosoi* in the foci. On the other hand, however, three cases[2),8),9)] of chromoblastomycosis confined to the brain and without any lesions elsewhere in the body related to the fungus have been reported. The present case is a 3 years 5 months boy who had shown clinical findings of a tumor of the liver with brain metastasis, and autopsy revealed chromoblastomycosis chiefly affecting the liver, the lymph nodes and the brain.

DESCRIPTION OF THE CASE

Case: A Japanese boy, 3 years 5 months old, was admitted to the Sennan Hospital on May 13, 1956.

Family History: A sister, 1 year old, and parents, farmers were in good health. No one in the family was known to have died of malignant tumors.

Personal History: He was born in full-term, with easy delivery, weighing

笹野伸昭，岡本豊三，高橋　隆，鈴木昭三

3,300 gr. at birth. He was brought up by mother's breast and apparently developed without trouble. No antibiotics including penicillin has been administered. Intelligence was seemingly maturing normally. He showed allotriophagic tendency. At about 1 year of age, he began to chew tobacco, then learned to smoke, and later on his consumption rose to 10 cigarettes a day. Since the beginning of March, 1956, he frequently complained of bellyache and was given anthelmintic on such occasions. At the end of April, dark-yellow urine was excreted, and he gradually lost spirit. Occasional fever and heavy nightsweats were complained every night. On May 13, 1956, he was brought to the Sennan Hospital, Miyagi Prefecture, with chief complains of fever above 38°C and nightsweats.

Findings at Admission : A medium sized, badly nourished boy was brought to the hospital. His skin was pale, dry and slack. Subcutaneous fatty and muscular tissues were ill-developed. He looked pale and anxious. No abnormal findings were observed on percussion and auscultation of the chest. Abdomen was found swollen. Liver was also swollen and palpable 4–5 fingers' breadth below the costal arch with a sharp-edged and smooth-surface. Spleen was not palpable. No accumulation of ascites was observed. Swelling of lymph glands of the body was not found. Tendon reflexes were within normal limits and no pathological reflex was seen. He was not jaundiced.

Laboratory Findings : Height, 93 cm. Body weight, 9,630 gr. Measurement around the abdomen, 60 cm. Erythrocytes count, 4,680,000. Leucocytes count, 21,400 ; eosinophiles, 54%; lymphocytes, 9%; neutrophiles with rod nuclei, 0%; lobular nuclei, 37%. Hemoglobin, 55% (Sahli). Icteric index, 11. Urine analysis; protein, positive in trace. Urobilinogen, negative. Gmelin, suspiciously positive. Eggs of ankylostoma and ascaris in feces were not found. Latent bleeding reaction was, however, positive. Liquor pressure on puncture of lumbar vertebra in lateral position, 100 mm of water. Neither luminous dust nor xantho chromia was found. Pandy's test ; showed negative test.

Patient's Course : After hospitalization, temperature continued to fluctuate around 38.5°C, appetite was utterly poor and heavy sweating was observed. Frequently he complained of stomach ache. No effect was noticed with administration of penicillin, chloromycetin, vitamin K and glucuronic acid solution administration on the liver. Conditions deteriorated day by day. On June 1, general tonic spasm, particularly severe on the right half of the body, appeared, accompanying accentuation of the right patellar tendon reflex and other pathological reflexes. On the next day, atonic paralysis appeared in the right half of the body. All consciousness was lost and the boy died on June 3, 1956.

Clinical Diagnosis : Cancer of the liver with metastasis to the brain.

Pathological Diagnosis :

1. Chromoblastomycosis.

a) Many dark-brown granulomas of fowl-egg size in maximum in the liver, especially in its left lobe.

b) Many similar granulomas of similar size in the brain.

c) Dark-brown granulomas of less than nut-size in lymph nodes in the mesenterium, inside the liver, around the stomach and in the posterior abdomen.

d) Granulomas of small-finger tip size at the bifurcation of the trachea and its surrounding tissues.

e) Dark-brown granulomatous ulcer of small bean-size in the caecal mucosa.

f) Microscopic granuloma of submiliary size in the right kidney.

2. Stricture of the choledochus due to granuloma in periportal lymph nodes and a granulomatous ulcer of small bean-size, on the mucous membrane inside the liver.

3. Mild general jaundice.

4. Catarrhal tracheitis and bronchitis.

5. Bilateral lacunal tonsillitis, etc.

Macroscropic Findings: An emaciated corpse of male infant. Height, 93 cm. Weight, 9,6 kg. Head was comparatively large and asymmetric. Occiput was slightly protruding, but no deficiency in skull. Skin was generally lightly jaundiced. The conjunctiva of the eye was also yellow-stained. No abnormal eczema, tumors and ulcers were observed on the skin.

Upon laparotomy, the liver was found greatly swollen. Its lower edge reached 3 cm to the left and 9 cm to the right mammary lines below the costal arch. Except a fibrinous adhesion of the gallbladder to the transverse colon, no pathological adhesion was observed. 130 cc of dense yellow ascites accumulated, containing some fibrine lumps. The diaphragm was slightly elevated.

Liver: Weight, 1,470 gr. The left lobe, a little larger than normal, was covered with dark brown finger-tip size nodes, uneven on the surface, and also on the cut surfaces, especially in the left lobe. The nodes were dark-brown and spherical, sometimes hard scars being found at the center and sometimes fused together into fowl-egg size. The surrounding parenchyma was found compressed and atrophied, and darker in its brown tone than that which is common at the boy's age. The central part of the lobuli was hyperemic, but neither picture of cirrhosis nor any sign of engorged liver was visible. Similar tumors in size up to nut-size were found in a large number within the liver, and this tumor formation was found spread to the lymph nodes in the ligamentum hepatoduodenale. In particular, the choledochus was found compressed, constricted and somewhat bent by a large tumor inside the liver near the duct, causing dilatation of the duct above it and the gallbladder. In the bile duct below it, a shallow small-bean size ulcer formed by granulomatous invasion from the surrounding lymph nodes was observed. The portal vein seemed to be free of compression

from any lymphadenoma. The lymph nodes in the mesenterium and the retroperitoneum, were also affected by dark-brown hard granulomas of nutsize or/and smaller, and had dark-brown parenchymatous or sometimes daisy-wise cicatrized granulomas in the center of the cut surfaces.

Brain: 1,400 gr. in total weight. It was excessively swollen, with shallow sulci and flattened gyri. In both the parietal lobes hard dark-brown spherical nodes were found, similar to those found in the liver, of which some reached the meningeal surface, showing rough surface. Softened foci surrounded it. Similar nodes were found in the left frontal lobe, in the right parietal and occipital lobe, and on the inside and the outside of the left occipital lobe. A tumor of mandel-size was found reaching from the left cerebral crus into the mesencephalon. Besides, one node of small finger-tip size was located in the cerebellar vermis. These nodes were of brown color and the larger ones showed relatively clear-cut boundaries between the surrounding cerebral parenchyma, but the smaller were less distinctly circumscribed.

The lungs were somewhat hyperemic but emphysematous. Though the boy smoked, macroscopic carbon-dust deposit was not much perceptible. Atelectasis was observed in the dorsal part, especially, of the right lung both in the upper and the lower lobes. Hyperemic swellings of finger-tip size at the largest were found and slight carbon deposit and dark-brown foci in a small number were observed in the intrapulmonary lymph nodes. Similar anomalies were found in the lymph nodes around the trachea. The thymus weighed 7 gr. Some parenchyma was contained, but it was rather atrophied, and rich in fiber, considering the boy's age.

Digestive Tract: The mucous membrane was found edematous and the small veins in it were dilated to some extent. The lymph follicles in the caecum was rather well developed. A small bean-size navel-form ulcer was found laying bare its dark-brown bottom at the tip of the caecum. Further details were omitted.

Histological Findings: In the granulomas in the liver, various sizes of coagulation necrosis were surrounded by infiltration of diversified cells including round mononuclear cells of varying size, leucocytes and histiocytes, sometimes intermingled with Langhans type or foreign body type giant cells, which were accompanied with growth of capillaries and heavy proliferation of fibers. The most notable phenomenon was that numerous yellow dark-brown staining small bodies were infesting the inside of the necrotic foci, especially, the vicinity of the granulomatous boundaries in the periphery. These were apparently the hyphae of the pathogenic fungi of chromboblastomycosis, which were often spherical in shape. The outer membranes of the hyphae were particularly dark-staining, so that a double outline was visible. Often a transverse partition was seen on the equatorial plane, showing the appearance of division and proliferation. The

size of these bodies was variable, the largest being several times as large as the erythrocytes, while the smallest smaller than the latter. Often they formed rosary-like chains or were of articulate hyphae, or sometimes showed branched arboreal arrangement. In general, the solitary round bodies were large and were black-brown, but when they were connected in rosaries or arboreally, they stained only light-yellow and the individual corpuscles were smaller. Such small bodies were seen in innumerable abundance everywhere in the necrotic lesions or small abscesses in the liver, but generally they were densely packed in the lesions where the necrotic substance was small and fresh, and contains many shreds or relies of dead nuclei. On the other hand they were apparently less abundant in the central part of the larger lesions with wide-spread necrosis but in their peripheral parts dark-staining corpuscles were more numerous. These corpuscles or hyphae were yellow or dark-brown, but were stained violet by Goodpasture method, red with PAS and in the color of Pyronin under Unna-Pappenheim staining. They proliferate in reaction granuloma or coagulation necrosis and invade the surrounding liver tissue through the sinusoids and the pericapillary lymph-space.

Similar coagulation necrosis and granulomas as well as similar innumerable small dark-brown bodies were found in the lesions in the lymph nodes.

The foci in the brain were generally fresher, and granulomatous cell reaction was observable centering on wide spread necrosis and blood vessels. Often small dark-brown bodies seen in the liver lesions were also prevalent here in large number. Here, the vascular walls were frequently swollen, the intima and the media also being thickened, and accompanying formation of thrombus, the small bodies were found locally, spreading to the outside of the blood vessels. These findings suggest a metastasis through blood-flow. The lesions were much more numerous and wide-spread than observed macrosopically, similar lesions being found more or less numerous in the mesencephalon, the pons and the medulla oblongata.

The small ulcer in the caecum had been formed by destruction of a granuloma originating in a lymph follicle. Innumerable small bodies, showing the varied stages from nearly colorless to yellow and to dark-brown coloration, were demonstrated here too.

Another small granuloma with dark-brown hyphae at the center was found in the cortex of the right kidney, indicating origination in hyphae embolism in a capillary of a glomerulus.

In the lungs, we found changes accompanying bronchitis, peribronchitis and aspiration pneumonia. Often carbon dust was deposited in the secretion of the bronchial mucosa, carbon-dust phagocytes were found wandering out into the alveolae and such deposits were found on the stroma tissue, too. No granulomatous lesion was perceptible.

DISCUSSION

Since Brumpt[3] first discovered a pathologic fungus of chromoblastomycosis, a strain named *hormodendrum pedrosoi*, many different strains have been isolated in many parts of the worlds, so that, in the present-day mycology, the nomenclature and classification concerning them are not unanimously settled, but the common name of Hormodendrum has been accepted by most mycologists. In this case, it was regrettable that cultivation was not carried out, but the characteristics shown by the yellow or yellow-brown spores and the hyphae found in the tissues were those of the fungi of the genus causing chromoblastomycosis.

The appearance of clinical cerebral symptoms was only three days before death, and the autopsy findings also showed that the granulomas and the hyphae in the brain foci were far fresher than those in the liver and the lymph node near by. Then, the next question is, what was the passage through which the chromoblastomycosis in the liver had been carried to the brain. The portal vein suggests itself immediately. The dark-brown ulcer found in the caecum was a very small focus formed by destruction of a mycotic granuloma in a lymph apparatus. They are like those found in the kidney, etc., and seemed to be due to a comparatively late dissemination by blood-flow. In other mycosis, the cases of primary occurrence in the lungs come most frequently into the question, but in this case no granulomas or brown bodies were perceptible in the lungs.

A more interesting fact concerning this case was that, in spite of his young age, the infant began to eat tobacco only one year after birth, and soon thereafter to smoke cigarettes. The findings of rather severe bronchitis, carbon dust phagocytes wandering out into the alveoli and the deposit of carbon dust on the pulmonary stroma for an infant 3 years 5 months old were sufficient to substantiate the above-mentioned smoking habit. It is known that chromomyci causing chromoblastomycosis often infest plants, but in manifesting such pathogenesis, the fungi act on the skin in most cases. Metastases to the internal organs are very rare. Most rarely the lesions are limited to the internal organs without skin manifestations, but such cases hitherto reported[2),8),9)] are confined to the isolated brain abscess. This is the first case ever reported of a chromoblastomycosis initially limited to the liver and showing no skin symptoms. The patient had no record of marked superficial trauma. This leads us to infer that the above-mentioned morbid appetite for cut tobacco and early habit of cigarette smoking of the child have caused disorder in his general conditions and facilitated the growth of the fungous hyphae usually ineffective as pathogenic factor in the inner organs.

SUMMARY

The authors reported the first case ever reported of chromoblastomycosis

primarily occurring in the liver without any dermal manifestations. The case, a boy 3 years and 5 months old, began to eat shred tobacco only around 1 year old and later on acquired the habit of smoking cigarettes. During about 3 months of the clinical course, the patient showed fever and swelling of the liver and died of cerebral symptoms. Autopsy revealed multiple yellow-brown granulomas chiefly in the liver and also in the lymph nodes in the abdomen and the thorax, and multiple metastasis to the brain. We failed in isolating and cultivating the pathogenic fungi, but the existence of chromobolastomycosis was ascertained, considering the histological features of the granulomas, and the characteristic form and color of the dark-brown small bodies or the hyphae found in the foci in innumerable abundance.

Acknowledgement

We are greatly indebted to the following persons for the diagnosis of this case. Dr. Yoshisada Takahashi, Professor of Dermatology, the Tohoku University School of Medicine; Dr. Masahiko Okudaira, Department of Pathology, The Tokyo University School of Medicine; Dr. Kenji Yamashita, Chief of Ear & Nose Clinic, the Kyoto National Hospital; and Dr. Shōji Ojima, Department of Pathology, the Kyoto University School of Medicine.

The principal findings of this case were reported[12] at the 47th Annual Meeting of the Japanese Pathological Society in April, 1958.

References

1) Barrack, B.B., Med. J. Australia, 1957, **1**, 189.
2) Binford, C.H. *et al.*, Amer. J. Clin. Path., 1952, **22**, 535.
3) Brumpt, E., Précis de Parasitologie. 3e éd., Masson, Paris, 1922. (cited from Takahashi[13]).
4) Carrion, A.L., Ann. New York Acad. Sci., 1950, **50**, 1255.
5) Carrion, A.L. & Koppisch, E., Puertorico J. Pub. Health & Trop. Med., 1933, **9**, 169.
6) Conant, N.F. *et al.*, Manual of Clinical Mycology. 2nd ed ., 262, Philadelphia, 1954.
7) Kano, K., Arch. f. Derm. u. Syphi., 1938, **176**, 282.
8) King, A.B. & Collette, T.S., Bull. Johns Hoskins Hosp., 1952, **91**, 298.
9) McGill, H.C. & Brueck, J.W., Arch. Path., 1956, **62**, 303.
10) Mead, M. & Ridley, M.F., Med. J. Australia, 1957, **1**, 192.
11) Nishi, M., Tr. Soc. Path. (Jap.), 1956, **45**, 566.
12) Sasano, N. *et al.*, Acta Path. Jap., 1958, **8**, 447; Tr. Soc. Path. (Jap.), 1958, **47**, 400.
13) Takahashi, Y., Jap. J. Derm. Urol. (Jap.), 1937, **41**, 153 & 225.

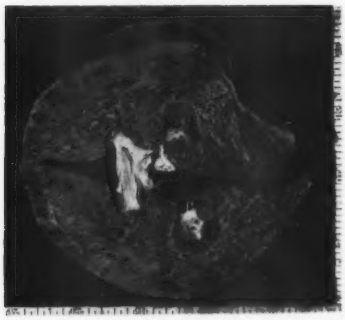


Fig. 1. Cut surface of the liver. Innumerable dark-brown granulomas in multiple existence, particularly dense in the left lobe. A goose-egg size fused node centering on the portal vein between the two lobes.

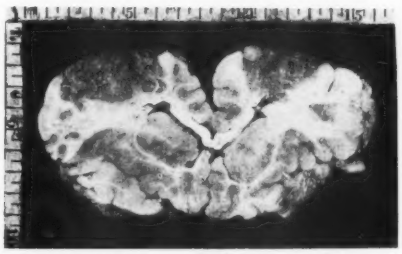


Fig. 2. Frontal section of the brain. Dark-brown ill-defined granulomas of goose-egg size at the largest found in the parietal lobes, especially in the cortex there.

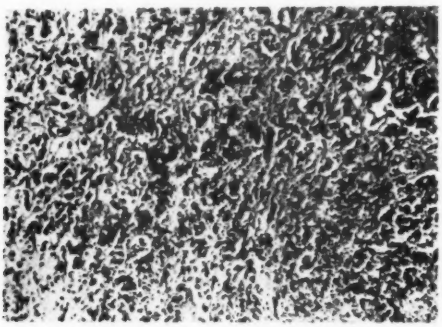


Fig. 3. A part of a large granuloma in the liver. Innumerable dark-brown small bodies in granulation tissue. Right half, forming an abscess. In the left half, dark-brown small bodies invading the remnant liver tissue (Hematoxylin; low magnification).

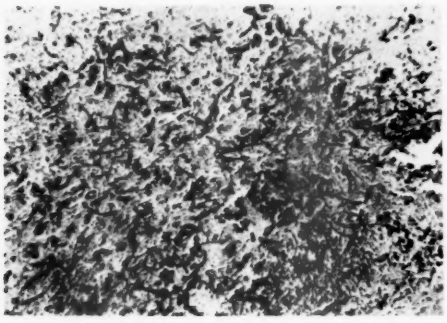


Fig. 4. Innumerable hyphae found in a granulomatous lesion in the liver, as if in pure culture, and varied in form (Goodpasture; low magnification).

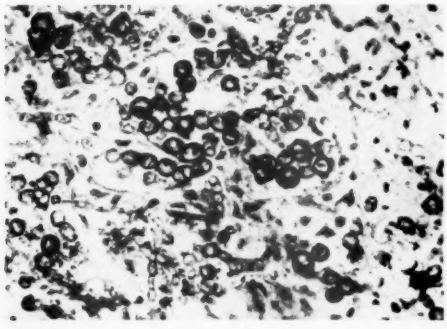


Fig. 5. Dark-brown small bodies in a granuloma in the liver, with thick membrane showing as rings and sometimes equatorial partitions. In solitary existence or in groups, and sometimes as articulate hyphae in rosary-like series (Hematoxylin; high magnification).

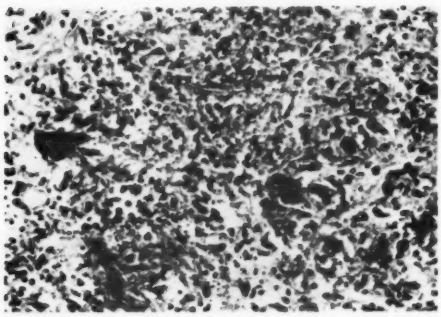


Fig. 6. Langhans type giant cells and dark-brown corpuscles found in a granuloma in a periportal lymph node (Hematoxylin and eosin; medium magnification).

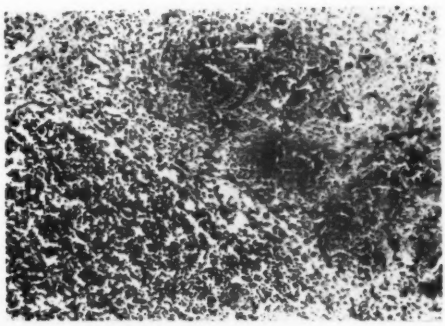


Fig. 7. Necrotic granuloma in the ulcer in the caecum. Numerous dark-brown small bodies. On the lower left, the remainder of lymph apparatus visible (Hematoxylin and eosin; low magnification).

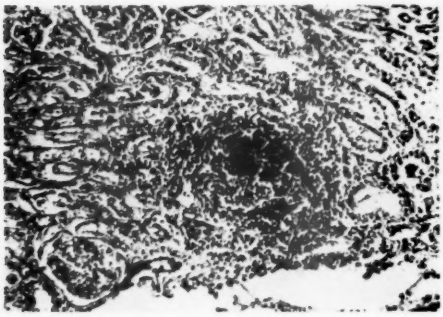


Fig. 8. A part of the renal cortex. Embolism and a small granuloma surrounded the proliferated dark-brown small bodies in a capillary of a glomerulus (Hematoxylin and eosin; low magnification).

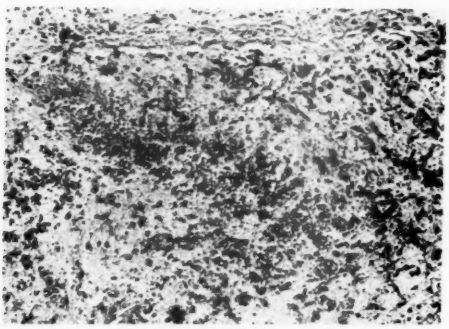


Fig. 9. A part of the cerebral cortex. Necrosis by dark-brown small bodies, granulomatory thickning of the pia mater observable (Hematoxylin and eosin; low magnification).

Tohoku J. Exper. Med., 1961, 73, 191–199

Origin of Hippocampal Seizure

By

Norio Taira

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, September 6, 1960)

INTRODUCTION

Since Gibbs and Gibbs' experiment[1] it has been well known that the hippocampus is particularly prone to produce seizure discharge upon stimulation thereof and its related structures, and many investigators[2-6] have worked on this problem. Nevertheless, what is responsible for the liability of the hippocampus to seizure discharge remains as an open question[7]. To answer this question it may be profitable to clarify what structures of the hippocampus are involved in seizure discharge. For this purpose a laminar analysis of seizure discharges was carried out in the hippocampus in the present experiment.

On the other hand, Suzuki[8] has correlated evoked responses to laminar stimulation of the hippocampus with its structure. To assess the finding obtained from the laminar analysis and to get further information about the origin of seizure discharge the latter was made to interact with the evoked responses to laminar stimulation of the hippocampus.

METHOD

Non-anesthetized rabbits immobilized with succinyl choline chloride were used. Surgical and experimental procedures were almost the same as those described by Suzuki[8] except for the following.

For initiating a seizure discharge a stimulating electrode of Granit's type was inserted into a depth of 0.3–0.8 mm. below the ependymal surface, the point of insertion being at a distance of a few millimeters from an ependymal recording electrode. Through the stimulating electrode a train of negative pulses of 1–2 msec. duration and of 20–100 μA. was delivered monopolarly at a frequency of 10–100 per sec. for 2–3 sec. Such stimuli were applied at intervals of 10–15 min., which was long enough to obtain a conspicuous seizure discharge each time.

Long recordings of seizure discharges were made with an inkwriter, and in addition an oscilloscope was used to record evoked potentials and individual potentials of the seizure discharge. RC amplifiers with a time constant of 0.01–

平　則夫

1.5 sec. were used. However, they were found to be incovenient to observe electrical phenomena immediately following seizure eliciting stimuli because they were blocked for a considerably long time by shock artefacts (cf. Figs. 3, 4 and 6). To avoid this disadvantage RC amplifiers with Miller's integrator[9] were used in later experiments (cf. Fig. 5).

RESULTS

1. *Patterns of seizure discharge*

Tonic and/or clonic seizure discharges such as those described by previous workers[2-4),6)] were easily elicited by the method of stimulation in the present experiment. Typically a seizure discharge began with a tonic pattern immediately following stimuli, changed into a clonic one and subsided gradually, coming to an end in scores of seconds[10)]. In the tonic phase seizure potentials were discharged usually at a rate of 20–40 per sec. In most cases the clonic pattern was more marked, but the tonic one could prevail in some cases. After a seizure discharge initiated by stimuli had subsided, a new seizure discharge appeared spontaneously in some cases.

2. *Laminar distribution of seizure discharge*

Exploration of the laminar distribution of seizure discharge was carried out as follows: Inserting a steel needle electrode into depths from the ependymal surface in steps of 0.1 mm. during a seizure discharge a record of the discharge was taken with the inkwriter at each position of the needle electrode, where a surface record taken at a point adjacent to the entry of the depth electrode served as control. In Fig. 1 are shown typical records (upper depth record and lower

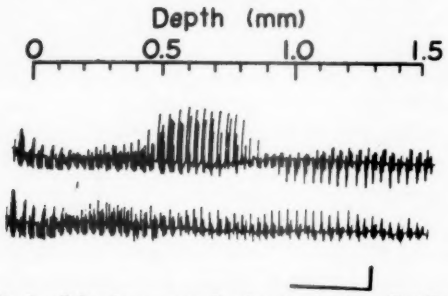


Fig. 1. Inkwritten records showing laminar distribution of seizure discharge at depths (upper record) and monitor at ependymal surface (lower record). Depths from ependymal surface are indicated by scale. In this and all subsequent figures upward deflection of both inkwritten and oscilloscope records denotes negativity. Time and voltage calibrations are 5 sec. and 1 mV. respectively.

surface one) obtained for a seizure discharge of clonic pattern in such experiments. As clearly seen in this figure, the negative potentials were increased in amplitude somewhat abruptly at a depth of 0.4–0.5 mm. from the ependymal surface, showed the maximal amplitude in a range of 0.5–0.8 mm., and then decreased rapidly in amplitude until it became zero-isopotential at a depth of 0.9 mm. With further advancement of the electrode the potentials reversed their polarity to become positive. In general the amplitude of the positive potential was smaller than that of the negative one.

Such continuous records as shown above could not be obtained for a seizure discharge of tonic pattern because it disintegrated rapidly into that of clonic pattern.

A depth of 0.5–0.8 mm. where the seizure discharge attained a maximal negativity corresponds to the level of the somata and apical dendrites of the hippocampal pyramids[8),11)]. Thus, the finding described above indicates that the somata and apical dendrites of the hippocampal pyramids become a major sink during the seizure discharge.

3. *Interaction between seizure discharge and evoked potentials*

To provide further evidence for the statement described above and to get information about the mechanism of initiation and subsidence of the seizure discharge some experiments were done on the interaction between the seizure discharge and the evoked responses to laminar stimulation of the hippocampus.

Depending upon the lamina stimulated, Suzuki[8)] obtained three kinds of evoked potential, that is, a slow spike potential (SP), slow wave (SW) and wave and spike-like potential (WSP). The three kinds of evoked potential are shown schematically in Fig. 2. The SP was obtained by stimulation of the alveus, the SW by stimulation of the level of the basal dendrites and the WSP by that of

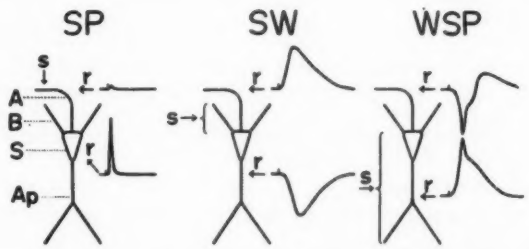


Fig. 2. Types of evoked potentials elicited by laminar stimulation of hippocampus. Arrows denoted by s indicate loci of stimulation. Arrows denoted by r indicate loci of recording. A: Axon. B: Basal dendrite. S: Soma. Ap: Apical dendrite.

the somata and apical dendrites of the hippocampal pyramids. The SP was assigned by Suzuki[8] to the antidromic activation of the somata and proximal dendrites of the hippocampal pyramids, the SW to the postsynaptic activity of the basal dendrites, and the WSP to the orthodromic activation of the somata and apical dendrites.

A single stimulating pulse was applied to — say the alveus — at intervals of about 2 sec. to obtain a certain kind of evoked potential — say SP, and then the seizure eliciting tetanizing stimulus was given to make the evoked potential interact with the seizure discharge so elicited. Generally speaking, every kind of evoked potential was affected more or less by the seizure discharge. When the seizure discharge had subsided it happened sometimes that an evoked potential gave rise to a new seizure discharge of abortive form. This may be compared to the similar observation by Rosenblueth and Cannon[2] on the neocortex.

a) *Interaction between seizure discharge and slow spike potential (SP)*

The SP was affected only by the seizure discharge of high frequency or tonic seizure discharge. This is shown in Fig. 3 in which depth recording (the upper

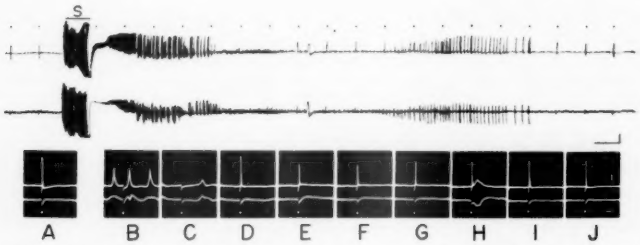


Fig. 3. Inkwritten and oscilloscope records showing interaction between seizure discharge and slow spike potential. In this and all subsequent figures upper lines of both inkwritten and oscilloscope records refer to depth electrode and lower ones to ependymal electrode. Dots above inkwritten records and those below oscilloscope records indicate delivery of single test stimulus for slow spike potential. A represents control responses before seizure discharge and B–J records obtained after delivery of seizure eliciting stimulus. S : Period of seizure eliciting stimulation. Time and voltage calibrations are 2 sec. and 1 mV. for inkwritten records, and 20 msec. and 1 mV. for oscilloscope records respectively.

lines of both inkwritten and oscilloscope records) was made at a depth of 0.4–0.6 mm. As clearly seen in this figure, the SP disappeared, and, instead, spike-like potential of seizure discharge appeared once the seizure discharge was initiated by electrical stimuli (Fig. 3 B and C). However, the SP recovered rapidly as soon as the seizure discharge was attenuated (Fig. 3 D). As can be seen in Fig. 3 G, H and I, the SP was not affected by a seizure discharge which occurred

spontaneously, probably because the frequency of this discharge was not so high as that of the evoked seizure mentioned above.

It may be observed that the SPs (E and F) suffered some reduction in amplitude as compared to the control (A) towards the end of the evoked seizure discharge.

b) *Interaction between seizure discharge and slow wave (SW)*

Once the seizure discharge started the SW was abolished completely and replaced by potentials of seizure discharge (Fig. 4 B and C). Even when the seizure discharge abated the SW was still suppressed (Fig. 4 D and E). The SW recovered gradually thereafter (F–K), and it took about 20–30 sec. for the complete recovery after subsidence of the seizure discharge. This is a striking contrast to the behavior of the SP which recovered very quickly (cf. Fig. 3).

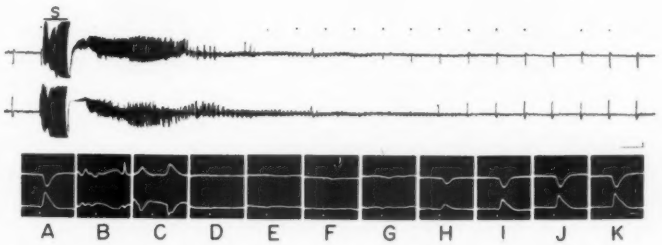


Fig. 4. Records as in Fig. 3 but for slow wave. Other conditions are same as in Fig. 3.

The polarity of the SW is negative at the ependymal surface and positive in the depth. Individual seizure potentials are opposite in sign to the SW (cf. A with B and C). It is to be noted that seizure potentials are always negative at the level of the apical dendrites of the hippocampal pyramids.

c) *Interaction between seizure discharge and wave and spike-like potential (WSP)*

A typical experiment on the interaction between the seizure discharge and the WSP is shown in Fig. 5. In this figure A represents control records of the WSPs taken at the level of the apical dendritic shaft and at the surface before the seizure discharge. The WSP in response to a strong stimulus has a spike-like potential superimposed on a negative wave (Fig. 6 A). The stimulus used in the experiment shown in Fig. 5 was not strong so that a negative wave without spike-like potential was obtained at the level of the dendritic shaft. In the initial phase of the seizure discharge WSPs of typical form with a spike-like potential appeared as if the intensity of the test stimulus had been increased (Fig. 5 B and C). In the course of seizure discharge the test response was affected,

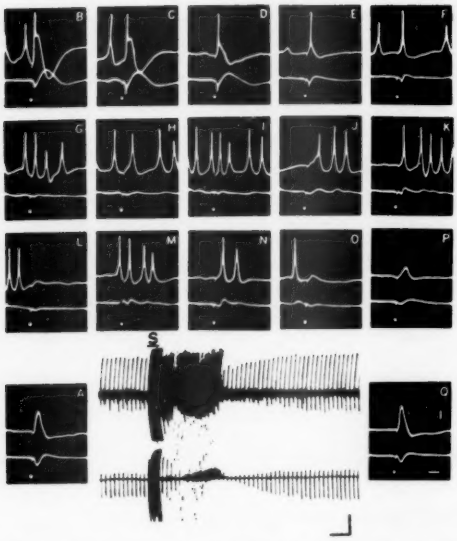


Fig. 5. Records showing interaction between seizure discharge and wave and spike-like potential. A and Q represent control records of wave and spike-like potential taken before and after seizure discharge. B–P are successive records taken since seizure discharge started. Time calibration is 10 sec. for inkwritten records. Other conditions are same as in Figs. 3 and 4.

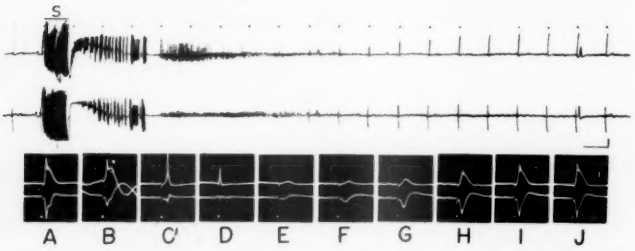


Fig. 6. Records as in Figs. 3 and 4 but for wave and spike-like potential. Other conditions are same as in Figs. 3 and 4.

but was restored towards the end of seizure discharge.

The recovery course of the WSP was similar to that of the SW, and it took about 20–30 sec. for the complete recovery after subsidence of the siezure discharge (see Fig. 6).

As has been described above, seizure discharges consist of negative spike-like and/or wave potentials when recorded at the level of the somata and apical

dendrites of the hippocampal pyramids. Seizure potentials can coexist with the spike-like component of the WSP and enhance the latter, while the SW is abolished completely by the seizure discharge.

DISCUSSION

Some active participation of the apical dendrites of the hippocampal pyramids in the seizure discharge was first pointed out by Euler *et al.*[12] They observed that the dendritic layer response to repetitive stimulation of the dorsal fornix became monophasically negative just before a seizure discharge broke through and that the seizure potentials evoked by such stimulation were also negative monophasic in the dendritic layer. In the present experiment the laminar recording has revealed that the seizure potentials were always negative and maximal in amplitude in the depths corresponding to the soma and apical dendritic layers of the hippocampus. This finding accords well with that by Euler *et al.*[12], and strongly suggests that the seizure discharge may be assignable to the activity of the somata and apical dendrites of the hippocampal pyramids.

The wave and spike-like potential (WSP) in response to stimulation of the soma and apical dendritic layers of the hippocampus has been assigned by Suzuki[8] to the activity of the somata and apical dendrites of the hippocampal pyramids by orthodromic invasion. Facilitation of the WSP by the seizure discharge implies that both share the same structure of the hippocampus. This fact lends support to the assumption that the main locus of seizure discharge is the somata and apical dendrites of the hippocampal pyramids. A close resemblance in shape between the individual seizure potential and the WSP may provide another evidence for the assumption, and further suggests that the seizure dischage may be initiated by synatpic activation of the somata and apical dendrites of the hippocampal pyramids.

Now, the question may be raised as to what is responsible for the abolishment of the slow spike potential (SP) and slow wave (SW) by seizure discharges. Suzuki[8] has attributed the SP to the antidromic activation of the somata and proximal dendrites of the hippocampal pyramids. Furthermore, he has reported that the SP interacted with the spike-like potential of the WSP, being occluded for about 20 msec. If the nature of the WSP is the same as that of the seizure discharge as has been assumed above, occulsion must be a cause of the abolishment of the SP by the seizure discharge. I am not in a position to account for the abolishment of the SW by the seizure discharge in the same way as above, for no information is available about the interaction between the WSP and the SW. Euler *et al.*[12] have observed DC potential shifts up to 3 mV. during the seizure discharge, where the surface layer becomes positive and the apical dendritic layer negative. The SW has been assigned to the postsynapitc activity of the basal dendrites of the hippocampal pyramids.[8] It is, therefore,

possible that a positive DC potential during the seizure discharge may lower the excitability of the basal dendrites and suppress the SW.

Postictal depression as observed for the SW and the WSP may be compared to that of the spontaneous electrical activity[2-3),6)]. The finding that the SP or antidromic soma spike was free from such depression may be attributable to the circumstance that no synaptic process takes part in the SP. The other kinds of evoked potential involve synaptic processes which must be subjected to postictal depression. As possible synaptic mechanisms involved in depression two factors may be considered, that is, synaptic fatigue and development of some synaptic inhibitory process during the seizure discharge. Further experiments will, however, be needed to clarify the mechanism of depression and subsidence of the seizure discharge.

SUMMARY

Seizure discharges were investigated in the exposed dorsal hippocampus of the rabbit paralyzed with succinylcholine.

1. Tonic and/or clonic seizure discharges lasting about 10–20 sec. were elicited by direct electrical stimulation of the hippocampus. In the tonic phase of seizure discharge potentials were discharged at a rate of 20–40 per sec.

2. The seizure potentials showed the maximal amplitude in a range of 0.5–0.8 mm. below the ependymal surface, being always negative in sign in this range.

3. The slow spike in response to stimulation of the alveus was abolished only by the seizure discharge of tonic pattern and recovered rapidly when the seizure discharge abated.

4. The slow wave in response to stimulation of the level of the basal dendrites of the hippocampal pyramids was abolished completely by the seizure discharge and was depressed for a considerably long time after the seizure discharge had subsided.

5. The wave and spike-like potential in response to stimulation of the level of the somata and apical dendrites of the hippocampal pyramids was augmented by the seizure discharge at least in the initial phase of seizure, being attenuated towards the end of seizure discharge. The wave and spike-like potential suffered postictal depression.

6. From these findings it was concluded that the somata and apical dendrites of the hippocampal pyramids are activated in the seizure discharge.

The author wishes to thank Prof. K. Motokawa for his invaluable discussion and suggestion throughout the course of the experiment and the preparation of the manuscript.

References

1) Gibbs, F.A. & Gibbs, E.L., Arch. Neurol. Psychiat. Chicago, 1936, **35**, 109.
2) Rosenblueth, A. & Cannon, W.B., Amer. J. Physiol., 1942, **135**, 690.
3) Jung, R., Arch. Psychiat. Zeitschr. Neurol., 1949, **183**, 206.
4) Kaada, B.R., Acta physiol. scand., 1951, **24**, Suppl., **83**.
5) Green, J.D. & Shimamoto, T., Arch. Neurol. Psychiat. Chicago, 1953, **70**, 687.
6) Liberson, W.T. & Akert. K., EEG clin. Neurophysiol., 1955, **7**, 211.
7) Magoun, H.W., The waking brain. 1958, Springfield, Charles C Thomas.
8) Suzuki, H., Tohoku J. Exp. Med., 1960, **71**, 331.
9) Suzuki, H. & Taira, N., Jap. J. Physiol., 1958, **8**, 365.
10) Renshaw, B., Forbes, A. & Morison, B. R., J. Neurophysiol., 1940, **3**, 74.
11) Cragg, B. G. & Hamlyn, L. H., J. Physiol., 1955, **129**, 608.
12) Euler, C. v., Green, J. D. & Ricci, G., Acta physiol. scand., 1958, **42**, 87.

H
N
J
L